Expression of the rabbit uteroglobin gene in pre-implantation mouse embryos

---

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Master of Applied Science

at

Lincoln University

New Zealand

by

Sharon Lilian Bishop-Hurley

Lincoln University

1993

ERRATA

Page	Line	
3	14	...hepatitis B gene...
	25	...RNA polymerase II,...
4	7	...This is controlled, in part, by...
5	8	...primarily organized as...
	20	...we have become increasingly...
	25	...splicing, and methylation...
	26	...Lewin 1990), all of which...
	26	...controlling mRNA translation...
	30	...1988), it has not...
6	10	...but may include...
	15	...the large majority of...
	16	...cellular mRNA. The...
	16	...depends on its structure (Kozak,...
	23	...long, which is...
7	10	...with mRNA expression (Kozak...
	23	...development are directed...
9	1	...RNA. These are needed...
	4	...growing oocyte, reaching...
	24	...numerous cilia is swept...
10	25	...rat prostate...
	26	...1986) and prostate...
11	14	...The rabbit uteroglobin...
	29	...lung and oesophagus...
12	3	...control exist at the...
13	6	...In the first, general...
	13	...also contains...
14	14	...(Tris-HCl...
15	15	...0.01% xylene cyanol...
	29	...an O.D of 1.0...
18	6	...chosen to maximally separate the...
	9	...most similarly sized band of...

19	8	...Conversion of 5'-protruding...
	14	...each of the 4dNTP in...
	15	...mM Tris-HCl...
21	18	...efficiency of 6.8 x 10^6 colonies/μg...
22	26	...and a 3'-SV40...
53	32	...small insert fragments
60	15	...DH5α cells containing...
61	16	...with a spectrophotometer...
62	4	...Solutions were filtered through a sterile 0.22 μm filter (Cameo IIS, MSI, Westborough, MA, USA), using positive pressure; The first few millimetres were discarded and the remainder was aliquoted into...
	27	...1.6 $mgml^{-1}$ in HT6
65	6	...transferred through 2 ml of HT6...
79	26	...number of follicles capable of...
81	10	...Drosophila embryos...

Expression of the rabbit uteroglobin gene in pre-implantation mouse embryos

by

S. L. Bishop-Hurley

Microinjected DNA sequences under the control of a foreign promoter may be expressed as early as the two-cell stage of mouse embryonic development, provided the correct trans-acting factors are present. A frequently utilised reporter gene in these studies is the *Escherichia coli* (*E. coli*) LacZ gene, which can be placed immediately 3' to the promoter of interest and expressed in a mammalian system.

In this study a 3.6 kb gene fragment containing the *E. coli* LacZ gene, a 5'-flanking Kozak initiation sequence and 3'-flanking SV40 polyadenylation signal was isolated from plasmid p610ZA and ligated into the polylinker region of pSP65 to yield the plasmid pSPLacZ. Fragments of 3.3 kb (-3254/+9) and 0.4 kb (-385/+9) of uteroglobin (UG) 5'-flanking region were inserted 5' to the LacZ fragment in this pSPLacZ construct to yield the plasmids pUG3.3LacZ and pUG400LacZ.

Expression of β-galactosidase from these UG promoter regions was compared to p610ZA, which contains 0.3 kb of the 5'-flanking region of the mouse heat shock promoter (HSP70) and expresses β-galactosidase in 100% of embryos tested. While p610ZA was efficiently expressed at the 2-cell stage of mouse pre-implantation development, non-expression of pUG400LacZ and pUG3.3LacZ indicated that there is a specificity in promoter usage at this stage.

Keywords **gene expression; LacZ reporter gene; uteroglobin; mouse heat shock promoter; 2-cell embryo**

ACKNOWLEDGEMENTS

I would like to thank the following people, without whom this study would not have been possible.

My supervisor, Prof. David Bullock for teaching me the process of practising good science and my associate supervisor, Dr Jon Hickford for his help with the day to day problems and making time available.

My lab colleagues and friends who taught me all I know about molecular biology techniques. I especially would like to thank Ms Anne Hill and Dr Sami Damak for the time they spent teaching me the technique of microinjection for their patience when things were not going so well; OiWah Liew, my (lazy) neighbour for her excellent view on cloning and her constant good humour; to everybody else in the lab group for their friendship and good company.

I especially would like to thank my husband Gregory Bishop-Hurley for his endless support and encouragement during my Masters degree, especially during the final days of writing up. Thanks also to my parents and family for their support over the years.

Contents

List of Tables

List of Figures

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 General Introduction

Embryonic development entails both temporal and spatial programmes of gene expression. The final consequence of this is that in the mature organism, expression of particular sets of genes occurs in a tissue-specific manner (Kothary *et al.*, 1989). Important information on the regulation of eukaryotic gene expression has been examined intensely *in vitro* by expressing foreign DNA constructs in terminally differentiated cells. However for tissue specific genes, this has been hampered by the lack of suitable cell lines needed for their expression.

Results from studies suggest that microinjected DNA sequences under the control of a foreign promoter may be transiently suppressed as early as the 2-cell stage of mouse embryonic development at a time when its genome becomes transcriptionally active (Brinster *et al.*, 1982; Stevens *et al.*, 1989; Vernel *et al.*, 1992).

The aim of this project was to investigate whether embryos at this stage of development have the necessary factors present to express tissue specific genes, not normally expressed at this stage of development. Rabbit uteroglobin, a tissue specific gene was used as the promoter under study in these experiements.

The review of literature relevant to this study has been divided into three parts.

Part 1 Eukaryotic Gene Regulation, aims to give a background on how higher eukaryotes regulate gene expression.

Part 2 Gene Expression during Pre-implantation Mouse Embryos aims to review how gene expression is regulated at this level.

Part 3 Control of Uteroglobin, aims to give a background on the control of tissue-specific expression of uteroglobin.

1.2 Eukaryotic Gene Regulation

1.2.1 Transcriptional Control of Gene Regulation

The control of gene expression at the transcriptional level is an important control mechanism that operates during both embryonic development and in the terminally differentiated cells of eukaryotic organisms.

Promoters and Enhancers

In this review only the class II genes transcribed by RNA polymerase II (Lewin, 1990), will be reviewed. These genes are transcribed into messenger RNA (mRNA) which can then be translated into protein. The promoters and enhancers of these genes consist of short cis-acting DNA sequences (modules) which are in turn recognized and bound by specific proteins called transcription factors to either activate or repress transcription (Dynan, 1989).

The combination of the modules making up these promoter and enhancer elements and the types of transcription factors which bind to them allows genes to evolve a distinct pattern of transcriptional regulation which can be tissue-specific (Dynan and Tjian, 1985; McKnight and Tjian, 1986; Dynan, 1989; Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Latchman, 1990; Lewin, 1990; Pabo and Sauer, 1992). In promoters these modules are clustered mainly upstream of the transcription initiation site for mRNA synthesis.

A specific module called the TATA box acts as a positional element, specifying the start of transcription, 20-30 nucleotides downstream at the 'A' position (Corden *et al.*, 1980) of the transcription start site (Breathnach and Chambon, 1981; Dynan, 1989). Promoters not containing an obvious TATA sequence usually initiate transcription over several sites, this being common in many 'housekeeping' promoters eg hypoxanthine phosphoribosyl transferase gene (HPRT) and the dihydrofolate reductase gene (DHFR) (Dynan, 1986; Sazer and Schimke, 1986). *In vivo* deletions or mutations within the TATA sequence and/or transcriptional start site consensus sequence (5' PyPyCAPyPyPyPyPy 3') results in a reduction in transcription initiation frequency (Wasylyk *et al.*, 1980; Grosveld *et al.*, 1982) and/or use of alternate transcription initiation sites (reviewed by Nevins, 1983).

A group of heterogeneous upstream promoter elements (UPEs) located approximately 40-200 nucleotides upstream of the transcription initiation site control the frequency of transcription initiation (Lewin, 1990). Some of these sequences and the transcription factors which recognize them are common to a diverse group of genes eg. the CAAT ('CCAAT') and GC ('GGGCGG') boxes while others such as the metallothionein regulatory element or heat shock element (Berk, 1989) are gene specific.

The differences between promoter and enhancer modules are mainly operational (Sassone-Corsi and Borrelli, 1986). In contrast to promoters, enhancers are not orientation- or distance-dependent but nevertheless must still be on the same DNA molecule as the promoter (Banerji *et al.*, 1981; Fromm and Berg 1983; reviewed by Yamamoto, 1986). For example enhancers have been located at the 3' end of genes (eg. β-globin gene), within introns (eg. immunoglobulin gene) and even in the middle of a coding region as in the hepatitis B enhancer (reviewed by Hames and Glover, 1988). Enhancers are required for full activity of their cognate promoters (Gluzman and Shank, 1983). Like promoters, some enhancers are constitutively active in many cell types eg the SV40 enhancer, while others operate in a tissue specific manner and/or are active in a temporal fashion during development eg immunoglobulin, β-globin and insulin enhancers (reviewed by Hames and Glover, 1988).

Transcription Factors

Transcription factors have a modular structure consisting of a DNA-binding domain separate from the transcriptional activation region (Mitchell and Tjian, 1989; Latchman, 1990; Pabo and Sauer, 1992). This allows them to both bind to the cis-acting recognition sequences present in promoters and enhancers, and to interact with one another and/or RNA polymerase II to either activate or repress transcription (Jones, 1990). This not only offers the potential for finer control of transcriptional activity but also allows a broader range of responses to environmental cues. Many transcription factors are ubiquitous eg Sp-1, Oct-1 (reviewed by Hames and Glover, 1988) while others have a more restricted tissue distribution eg Oct 2. Indirect evidence correlates the presence of these tissue-specific transcription factors with transcriptional activation of the genes to which they bind eg. GHF1 (Bodner *et al.*, 1988) and Pit-1 (Ingraham *et al.*, 1988; Nelson *et al.*, 1988) are both pituitary-

specific transcription factors; C/EBP present in only the liver and fat cells, is expressed in the fetal liver on embryonic day 13 (Kuo *et al.*, 1990); Oct-2, is a lymphoid specific factor (Staudt *et al.*, 1988); hepatocyte nuclear factor 1 (HNF1) is a liver-specific transcription factor (Courtois *et al.*, 1987).

Regulation of Transcription Factors

Many processes in early development require the correct temporal and spatial regulation of gene expression. This in part is controlled by regulating transcription factors at the transcription and post-transcription level (eg. Hinkley and Perry, 1992). For example, Oct-2 (Staudt *et al.*, 1988) and C/EBP (Xanthopoulos *et al.*, 1989) are transcriptionally regulated, while CREB (Yamamoto *et al.*, 1988; reviewed in Berk, 1989) and the heat shock transcription factor (HSF; Sorger *et al.*, 1987; reviewed in Berk, 1989) are regulated at the post-translational level.

1.2.2 Other Factors Involved in the Regulation of Gene Expression

Interaction of Transcription Factors with Nucleosomal DNA

Within the eukaryotic nucleus, complex metabolic processes involving DNA occur on a chromatin template. Although the formation and maintenance of chromatin structures are essential for the compaction of DNA within the nucleus, transcription factors must still be able to gain access to the regulatory DNA sequences in order to initiate transcription (Hayes and Wolffe, 1992).

The fundamental repeating unit of chromatin is the nucleosome (Richmond *et al.*, 1984). Because the DNA is deformed when wrapped around the core histones within the nucleosome (Hogan *et al.*, 1987) this can repress transcription by preventing access of transcription factors to the DNA regulatory elements (reviewed Hayes and Wolffe, 1992). A large number of experiments have suggested that the association of regulatory elements with the core histone proteins prevents them from being recognized and bound by transcription factors (reviewed in Felsenfeld, 1992). In a few studies, the position of a nucleosome relative to a particular DNA sequence was manipulated both *in vitro* and *in vivo*. These experiments established that the precise position of a regulatory element in the nucleosome can have important consequences for its interaction with transcription factors (Lirch *et al.*, 1987; Workman and Roeder,

1987; reviewed in Hayes and Wolffe, 1992).

Nucleosomes are organized into looped domains of approximately 60 kb in length, and are bound to the nuclear matrix via an AT-rich sequence called the matrix associated region (MAR) (Gasser and Laemmli, 1987; Getzenberg *et al.*, 1991; Stein *et al.*, 1991; Romig *et al.*, 1992; reviewed in Gross and Garrard, 1987). Generally the looped domains of transcriptionally-active chromatin are decondensed and are localized near the nuclear periphery (Hutchinson and Wintraub, 1985), while transcriptionally repressed chromatin domains are primarily orgnaized as condensed chromosomal loops and are localized near the nuclear interior (reviewed in Gross and Garrard, 1987).

There appears to be a preferential association of MARs with transcribed regions (Stief *et al.*, 1989; Zenk *et al.*, 1989; von Kries *et al.*, 1990). For example, the ovalbumin gene in chicken oviduct cells and the α_2-microglobulin gene in rat liver were preferentially associated with the nuclear matrix only in those cells in which they were expressed (Getzenberg *et al.*, 1991). In addition several enzymes such as topoisomerase II, RNA polymerases and transcription factors eg. myc protein, the large T antigen of the SV40 virus and EIA from adenovirus (reviewed by Getzenberg *et al.*, 1991), are enriched within the MAR.

1.2.3 Post-Transcriptional Regulation of Gene Expression

In recent years, it has become increasingly aware of the importance post-transcriptional regulation, plays in regulating gene expression (reviewed by Gallie, 1991). Precursors to mRNA undergo several post-transcriptional modifications in the nucleus and these play an important role in regulating gene expression (reviewed by Gallie, 1991). This includes the presence of a 7-methyl-guanylic acid cap (5"-m^7G) structure, 3' polyadenylation [poly(A)] tail, pre-mRNA splicing, methylation (reviewed by Brown, 1990; Lewin, 1990) all which play a role in controlling its translation (reviewed by Hershey *et al.*, 1991).

Methylation

Although DNA methylation has been frequently correlated with gene inactivity (Buschavsen *et al.*, 1987; Yisraeli *et al.*, 1988), however it has not been clarified

whether this increased methylation is a cause or a consequence of gene inactivity (Selkar, 1990).

Introns

The presence of introns within transgenes has been demonstrated to play an important role in gene expression in transgenic mice (Brinster *et al.*, 1988; Palmiter *et al.*, 1991) despite having little effect in transfected cell lines (Brinster *et al.*, 1988). Brinster *et al.* (1988) and Palmiter *et al.* (1991) showed that when introns were present within a transgene, the level of expression in transgenic mice was consistently higher than the same transgene without the introns. The reasons for this are still unclear, but may be include the presence of controlling elements within the intron that are important to transcription, the relationship of gene splicing and mRNA stability, or a contribution to nucleosome phasing or some higher order DNA structure (Merlino *et al.*, 1991).

mRNA Structure

The scanning model for the initiation of translation accounts for the large majoriy of cellular mRNAs. The efficiency whereby mRNA is translated depends on the structure of its mRNA (Kozak, 1989; reviewed by Hershey, 1991). Important features of this include the presence of a 7-methyl-guanylic acid 'cap' at the 5' terminus, secondary and tertiary structure, the initiator codon and its surrounding sequences and the poly(A) tail at the 3' terminus.

The 3'-Poly(A) Tail

The majority of class II gene transcripts contain a 3' poly(A) tail, 40-100 residues long which is added post-transcriptionally to the mRNA. The purposes of the poly(A) tail in gene regulation is not very well understood. Of late, consensus has built up around 2 major hypotheses to its function. One body of opinion supports that the poly(A) tail is involved in mRNA stability (Aatwater *et al.*, 1990), whereas others support the hypothesis that it is implicated in the control of mRNA translation (Gallie, 1991), although additional functions such as nuclear processing and transport (Brawerman, 1981; Wickens and Stephenson, 1984) cannot be ruled out to date.

Polyadenylation affects translational efficiency (Munroe and Jacobson, 1990).

mRNAs with poly(A) tails are translated much more efficiently than those lacking the poly(A) tail (reviewed by Hershey, 1991). This is an important feature of translational regulation in oocyte maturation and early development where adenylation and deadenylation are occuring. An example is the translational activation of dormant tissue-type plasminogen activator mRNA during meiotic maturation of mouse oocytes (Vassalli *et al.*, 1987).

7-methyl-guanylic acid cap (5'-m^{7}G)

Nearly all mammalian cellular mRNAs are thought to be capped (reviewed by Hershey, 1991). The presence and accessibility of the 5'-m^{7}G cap structure to the translation initiation factor eIF-4F is correlated with mRNA strenght (Kozak, 1989).

1.3 Embryonic Development

Although embryonic development in the mammal is strictly initiated at fertilization, its success is dependent on both the successful implementation of genetic and developmental programmes contained within the oocyte and embryo itself and the interaction of the pre-implantation embryo with its environment, the maternal reproductive tract. This genetic and developmental programme is initiated during oogenesis. During oogenesis the oocytes grow to a stage where they can be stimulated to undergo meiotic maturation and ovulation. The stockpile of gene transcripts and translation products accumulated during this time, later direct development from ovulation up to the early 2-cell stage (reviewed by Johnson, 1981; Magnuson and Epstein, 1981; Davidson, 1986; Schultz and Heyner, 1992).

The molecular changes that occur during mouse oogenesis and early embryonic development is directed by 4 distinct programmes which are separately reviewed here:

1. an oocyte growth programme regulating the growth of the primary oocyte.
2. an oocyte maturation programme which allows the oocyte to resume and complete meiosis up to the stage of ovulation.
3. an activation programme which commences and directs the events of fertilization through to the first cleavage.

4. an embryonic programme commencing with transcription from the embryonic genome.

1.3.1 Oocyte Growth Program

Oogenesis in the mouse is initiated during embryonic development 8.5 days post coitum. The primordial germ cells differentiate from the cells of the primitive ectoderm and migrate to the genital ridge (reviewed by Wassarman, 1983; Hogan *et al.*, 1986). En-route and after colonization of the early gonad, these germ cells mitotically divide to become primary oocytes. Meiosis is then initiated, progressing to the diplotene stage of prophase I of the first meiotic division. They then remain in meiotic arrest until the time of ovulation when the preovulatory LH surge stimulates the oocytes to resume meiosis (reviewed by Hogan *et al.*, 1986). Five days after birth, these primary oocytes (12 μm diameter) are all in meiotic arrest. Morphologically they are surrounded by a single layer of somatic pre-granulosa cells and constitute the non-growing pool of primordial follicles (Peters and McNatty, 1980); which are the sole source of adult germ cells.

Every day a number of these primordial follicles enter the oocyte growth programme. Growth of these primary oocyte is continuous, ending either in ovulation of a matured oocyte (unfertilized egg) or degeneration (atresia) of the follicle and its oocyte.

During this growth phase the oocyte grows from a diameter of 12 μm to 85 μm (excluding the zona pellucida). Follicle cells also divide from a single layer of flattened cells to 3 layers of cuboidal granulosa cells (~900 cells).

A gap-junctional mediated syncytium is formed between the oocyte and granulosa cells, allowing metabolic coupling to occur. This is essential for oocyte growth (Heller *et al.*, 1981); the metabolic transfer (Heller and Schultz, 1980; Moor *et al.*, 1980; Heller *et al.*, 1981; Colonna and Mangia, 1983) regulates a number of oocyte functions including growth (Buccione *et al.*, 1987), meiotic progression (Eppig and Downs, 1988) and protein phosphorylation (Colonna *et al.*, 1989). During this growth the oocyte synthesizes and accumulates macromolecular stores of protein, tRNA, rRNA, mRNA, polyadenylated [poly(A)] RNA and low molecular weight

RNAs. These are needed to later direct the oocyte during maturation, fertilization and the early stages of embryogenesis (reviewed by Telford *et al.*, 1990). The zona pellucida, an extracellular material composed of three proteins, ZP1, ZP2 and ZP3 is synthesized and deposited around the growing oocyte reaching a thickness of ~7 μm.

1.3.2 Oocyte Maturation Phase

During meiotic maturation all transcription activity ceases. A process of deadenylation, adenylation and degradation of maternal mRNA is initiated resulting in a net loss of ~30% of the polyadenylated mRNA pool between the fully grown oocyte and ovulated oocyte stages (Sternlicht and Schultz, 1981; Piko and Clegg, 1982; Bacharova and Paynton, 1988; Paynton *et al.*, 1988). In addition major changes in the overall pattern of protein synthesis occurs (Bower *et al.,* 1981; Wassarman, 1983), as well as major reorganization of organelles such as mitochondria, lysosomes and cortical granules (Wassarman, 1983). This 'maternal' program of gene expression is controlled by translation of pre-existing mRNAs. Vassalli *et al.* (1989) found that adenylation and degradation of maternal mRNA played a role in this post-transcriptional control of gene expression.

These changes occurring at oocyte maturation are necessary to allow progression through the first meiotic division and to maintain arrest of meiosis at the second meiotic division. They indicate that translation, deadenylation and adenylation are triggered by precisely timed developmental events (Vassalli *et al.*, 1989).

At this stage the first meiotic division takes place. One set of homologous chromosomes, surrounded by a small amount of cytoplasm is extruded as the first polar body. Each oocyte, surrounded by its zona pellucida and mass of granulosa cumulus cells is ovulated and through the action of numerous cilia are swept into the infundibulum of the oviduct (reviewed by Hogan *et al.*, 1986).

1.3.3 Activation Programme

At fertilization, the zygote enters an activation program which is to some extent a continuation of events initiated during maturation and independent of fertilization. Even if fertilization does not occur, the egg still manifests essentially the same changing qualitative pattern of proteins synthesized, but it takes 2-3 times longer to

complete (reviewed by Dworkin and Dworkin-Rastl, 1990).

Fertilization, DNA replication in the 1-cell zygote, cleavage to the 2-cell stage and the qualitative changes in polypeptide synthetic profile accompanying these events seem to be regulated largely, if not exclusively at the post-transcriptional level.

1.3.4 Embryonic Genome Activity

Zygotic gene activation occurs in 2 stages in the mouse, a limited activation occurring between 18-21 hours post-insemination (hpi) and a major activation occurring between 26-29 hpi which correspond to G1 and early G2 of the second developmental cell cycle (Flach *et al.*, 1982; Bolton *et al.*, 1984). Minor activation of transcription codes for the heat shock proteins 68 and 70 (Bensaude *et al.*, 1983). This is then followed by DNA replication and expression of a major set of embryonic genes during G2 at the mid 2-cell stage (Flach *et al.*, 1982; Bolton *et al.*, 1984). Some of these proteins are similar to the maternally derived transcripts while many others code for new developmental proteins.

Major activation of the embryonic genome is followed by a progressive decline in the expression of maternal mRNA between 26 and 47 hpi (Piko and Clegg, 1982). It is likely that this decline is due to degradation rather than deadenylation (Graus *et al.*, 1985).

1.4 Uteroglobin

1.4.1 Uteroglobin: Structure and Function

Uteroglobin (Beier, 1968) also called blastokinin (Krishnan and Daniels, 1967) is a small globular protein, consisting of 2 identical 70 amino acid peptides covalently linked by 2 disulfide bridges (Postingle *et al.*, 1978). Initially it was thought to be a protein unique to the rabbit, however uteroglobin-like proteins have also been reported in rat prostrate (Aunukker *et al.*, 1985), lungs (Lopez de Haro, 1988), human uterus (Cowan *et al.*, 1986) and prostrate (Manayak *et al.*, 1988).

Although the physiological role of uteroglobin has not yet been defined, its regulation by steroid hormones has made it a useful model for the hormonal control of mammalian gene expression, which varies with the tissue in which the gene is expressed. Uteroglobin is synthesized and secreted from the rabbit endometrium at a high rate during the pre-implantation stage of pregnancy under the control of progesterone and to a lesser extent estrogen (Beier 1968; Beato *et al.*, 1983; Westphal 1986). It is also synthesized in other tissues of the rabbit, under the control of different steriod hormones: estrogens in the oviduct (Kay and Feigelson, 1972; Goswami and Feigelson, 1974); androgens in the male epididymis (Lopez de Haro *et al.*, 1988); constitutively expressed (Savouret *et al.*, 1980) or weakly modulated by glucocorticoids (Torkkeli *et al.*, 1978; Fernandez-Renau *et al.*, 1984) in the lungs in males as well as normal and pregnant females.

1.4.2 Gene Structure

Rabbit uteroglobin gene is a single copy gene. It is 3 kb long and contains 2 introns and 3 exons (Bailly *et al.*, 1983; Suske *et al.*, 1983). An estrogen receptor binding site has been detected in the proximal promoter region between -265 and -253 from the start of transcription (Slater *et al.*, 1990). In addition progesterone response elements (PRE) and glucocorticoid repsonse elements (GRE) were detected far upstream from the transcription initiation site (Cato *et al.*, 1984; Jantzen *et al.*, 1987).

The locations of these binding sites correlate with 3 DNase I hypersensitive regions found in chromatin of hormonally stimulated endometrium which disappears after hormone withdrawal.

The uteroglobin gene of the mouse is as yet uncharacterized. However the gene for a rat homologue of rabbit uteroglobin, originally designated as 10 kDa Clara cell secretory protein (CC10) has been isolated (Hagan *et al.*, 1990), and cross-hybridizes with the homologous mouse mRNA but not with the rabbit uteroglobin mRNA (Hagan *et al.*, 1990). Like rabbit uteroglobin, rat uteroglobin mRNA is expressed both in lung and esophagus, as well as in the uteri of estrogen- and progesterone-treated rats. Expression in the uterus was lower than in the rabbit however (Hagen *et al.*, 1990).

1.5 Summary

The control of gene expression in eukaryotes is a complex process that is just starting to be unravelled. Many different levels of control exist a the transcription and post-transcription level and the potential importance of these in regulating gene expression may vary from gene to gene.

CHAPTER 2

PLASMID CONSTRUCTION AND CLONING

2.1 General Introduction

The purpose of the experiments described in this chapter were to manipulate plasmid DNA to produce recombinant DNA for microinjecting into mouse zygotes. This chapter is divided into two parts. In the first general procedures common to most experiments are described while in the second, details of the cloning experiments are given. Finally the results of the experiments are described and discussed.

2.2 Materials

2.2.1 Vector DNA - pSP65

Vector pSP65 provided by Dr J. H. Hickford was originally purchased from Promega Biotechnology. It contains a phage SP6 promoter subcloned into the pUC12 polylinker site. The 3005 bp plasmid also contained the ampicillin resistance gene (*bla*), encoding β-lactamase.

2.2.2 Insert DNA - p610ZA

Plasmid p610ZA (6530 bp) was provided by Dr M. Kennedy, Laboratory of Molecular Biology, Cambridge and purified on a caesium chloride density gradient by Dr A. Gooneratne at Lincoln University. It consists of 300 bp (-86/+221) of 5'-flanking mouse heat shock promoter 70 (Hsp 70) fused in frame to codon 8 of the 3600 bp *Escherichia coli* (*E. coli*) LacZ gene (Casadaban and Cohen, 1980), cloned into the Xba I site of the pUC13 polylinker region. Expression of β-galactosidase from this LacZ reporter gene in eukaryotes, was aided by the presence of a 5'-flanking translation initiation sequence (Kozak, 1984) and 3'-flanking SV40 polyadenylation signal. The plasmid also contained the ampicillin resistance gene (*bla*), encoding β-lactamase.

2.2.3 Insert DNA - pUG400

Plasmid pUG400 (4766 bp) was provided by Professor D. W. Bullock, Centre for Molecular Biology, Lincoln University. It consists of the 403 bp (-394/+9) Bam HI fragment of 5'-flanking rabbit uteroglobin (UG) DNA (UG400) cloned into the Bam HI site of pBR322. The plasmid also contained the ampicillin resistance gene (*bla*), encoding β-lactamase.

2.2.4 Insert DNA - pUG3.3

Plasmid pUG3.3 (6222 bp) was provided by Professor D. W Bullock, Centre for Molecular Biology, Lincoln University. It consists of 3300 bp (-3254/+9) of 5'-flanking rabbit uteroglobin (UG) DNA (UG3.3) cloned into the Eco RI polylinker site of pSK Bluescript (Stratagene, La Jolla CA). The plasmid also contained the ampicillin resistance gene (*bla*), encoding β-lactamase.

2.2.5 T4 DNA Ligase

Bacterophage T4 DNA ligase and its 10X buffer (Tris-HCL, 660 mM $MgCl_2$, 50 mM; dithioerythritol, 10 mM; ATP, 10 mM; pH 7.5 @ 20 °C.) were purchased from Boehringer Mannheim (BM). One Weiss unit (Weiss *et al.,* 1968) corresponded to 0.2 units of bacteriophage T4 DNA ligase as determined in the exonuclease III resistance assay (Modrich and Lehmann, 1970).

2.3 Methods

2.3.1 TE buffer (pH 8.0)

TE buffer (10 mM Tris.Cl, pH 8.0; 1 mM EDTA, pH 8.0) was used to dissolve the DNA, because the EDTA binds the magnesium ions; a cofactor for most nuclease activity.

2.3.2 Restriction Endonuclease Cleavage

Restriction endonucleases purchased from New England Biolabs and Boehringer Mannheim, were used according to the manufacturers specifications. They were then deactivated by heating the DNA solution to the temperature specified in Table 3.1.1 of Bloch and Bartos (1992) for 15 minutes and the salt-containing buffer removed by

ethanol precipitation (Sambrook *et al.*, 1989). Cleaved DNA fragment(s) were electrophoresed on a horizontal agarose gel to monitor both the completeness of the restriction endonuclease reaction and confirm DNA fragment length (Sambrook *et al.*, 1989).

2.3.3 Agarose Gels Electrophoresis

Horizontal agarose gels were prepared and the DNA electrophoresed according to the methods of Sambrook *et al.* (1989). In these experiments both SeaKem^{R} Le Agarose (FMC Bioproducts, USA) and Bacteriological Agar (Gibco, UK) were used and the percentage agarose (w/v) was chosen to maximally resolve the DNA fragment(s) of interest (Table 6.1, Sambrook *et al.*, 1989). Tris Borate Electrophoresis (1X TBE) buffer used at a 1:10 dilution (0.09 M Tris-Borate; 0.002 M EDTA, pH 8.0) of the concentrated 10X stock and ethidium bromide at a concentration of 0.25 μgml^{-1}, were incorporated both into the agarose gel and electrophoresis buffer.

DNA samples were loaded onto the agarose gel with 0.1 volume of sucrose loading dye (0.4 M urea; 5% sucrose; 0.01% bromophenol blue; 0.01% xylene cyanate; 25 mM EDTA) which both increased the sample density and enabled the DNA to be tracked through the agarose.

In these experiments agarose gels were routinely used, both for the estimation of DNA concentration and confirmation of DNA fragment sizes (Sambrook *et al.*, 1989). The molecular weight standard used for these calculations was a lambda bacteriophage HindIII DNA digest (λHind III; Boehringer Mannheim) whose fragment sizes have been accurately determined (Daniels *et al.*, 1980). DNA was visualized over an ultraviolet (UV) transilluminator (UVP, Inc.) and photographs were taken using Polaroid professional film (Polaroid 667).

2.3.4 Spectrophotometric Determination of the Purity and Amount of DNA

The A_{260}/A_{280} ratio of isolated plasmid DNA was quantified on a UV spectrophotometer (Shimadzu^{R} UV-120-02) by taking readings at wavelengths of 260 nm and 280 nm, according to the methods of Sambrook *et al.* (1989). It was assumed that an O.D of 1.o corresponded to 50 μgml^{-1} of double-stranded DNA.

2.3.5 Phenol:Chloroform Extraction

Redistilled phenol was pre-equilibrated with TE (pH 8.0), according to the methods of Sambrook *et al.* (1989). DNA-containing solutions were deproteinized with 1 volume of phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1), according to the methods of Sambrook *et al.* (1989). This was repeated until no denatured protein was visible at the interface between the organic and aqueous phases. For solutions containing a low concentration of DNA (ie. <1 μgml^{-1}), its recovery was improved by back-extracting the organic phase and its interface with an equal volume of TE buffer (pH 8.0).

The DNA was recovered by precipitation with 2.5 volumes of ice-cold 100% ethanol, containing 0.1 volume of 3.0 M sodium acetate (pH 5.2) to aid recovery (Sambrook *et al.*, 1989). In solutions containing a low concentration of DNA (ie. <1 μgml^{-1}), its recovery was improved by precipitating for several hours at -20 °C before centrifuging on a benchtop microfuge (Biofuge A, Heraeus Sepatech, Germany) for 15-20 minutes at 13000 g and 4 °C. The pellet of DNA was washed with 1 ml of 70% ethanol to solubilize any salt and organic molecules present and the DNA recovered by centrifuging at 4 °C in a microfuge (Sambrook *et al.*, 1989). Trace ethanol was evaporated using a Speedvac Concentrator (Savant Instruments Co; Model RH 40-11, USA).

2.3.6 Preservation of Stock Cultures

E. coli strains were stored in the presence of 50% v/v glycerol to reduce damage by ice crystals. Long term storage of *E. coli* was at -100 °C while stort term stocks were maintained at -20 °C.

2.3.7 Miniprep Method using Alkaline Lysis

An aliquot of a frozen 50% glycerol stock of *E. coli* (containing the plasmid of interest) was streaked across an Luria-Bertani (LB) agar plate (Sambrook *et al.*, 1989), supplemented with 60 μgml^{-1} ampicillin. This was then incubated for 16 hours at 37 °C to obtain bacterial colonies. A single colony was picked from the plate and grown in a 15 ml polypropylene tube (Falcon^R 2096), containing 10 ml LB medium (Sambrook *et al.*, 1989), which was supplemented with 60 μgml^{-1} ampicillin. This was shaken at 225 rpm @ 37 °C for 17-20 hours. To maximize the quantity of

plasmid DNA harvested, the *E. coli* cells were collected by centrifugation when they were visually assessed to have entered the stationary phase of their growth (Sambook *et al.*, 1989). Plasmid DNA was isolated by alkaline lysis, using a protocol modified by Sambrook *et al.* (1989) derived from the methods reported by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

To ensure complete deproteinization, the DNA-solution was extracted with 1 volume of phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1) (Section 2.3.5). The DNA was recovered by precipitation with 2.5 volumes of ice-cold ethanol, according to Sambrook *et al.* (1989). RNA in this protocol was selectively degraded using 40 $\mu g ml^{-1}$ DNase-free RNase A (Boehringer Mannheim) in TE (pH 8.0) and incubated at 37 ^{0}C for 1 hour.

The identity of the isolated plasmid was verified with the appropriate diagnostic restriction endonuclease(s) and the DNA fragments analysed on an agarose gel. Because plasmid DNA isolated using this procedure are frequently contaminated with phenol and enzymes used in their isolation, they could not be accurately quantified using the spectrophotometer. DNA concentration was therefore estimated on an agarose gel.

2.3.8 Electrophoresis onto DEAE-cellulose membrane

DNA fragment(s) to be isolated were separated by size using electrophoresis through an agarose gel of the appropriate concentration. SeaKem^R Le Agarose, (a high quality agarose), was used to electrophoretically separate the DNA fragment(s), as other agarose preparations may contain inhibitors of DNA ligase. Electrophoretic recovery of DNA fragment(s) from agarose gels was adapted from the method of Sambrook *et al.* (1989). DEAE-cellulose membrane (Schleicher and Schwell NA 45) was used in preference to the dialysis membrane as originally reported by Girvitz *et al.* (1980). The DNA was eluted from the DEAE-cellulose membrane using a high salt elution buffer (50 mM Tris.Cl pH 8.0; 1 M NaCl; 10 mM EDTA pH 8.0) and extracted with 1 volume of phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1) and precipitated in ethanol using 0.2 volumes 10 M ammonium acetate and 2.5 volumes of ice-cold absolute ethanol (Sambrook *et al.*, 1989).

To confirm that only the desired DNA fragment(s) was purified, a small aliquot of the DEAE-purified DNA was electrophoresed on an agarose gel, containing agarose at a different concentration (w/v) from that used for the isolation of the DNA fragment(s). Because there is a linear relationship between the logarithm of the DNA electrophoretic mobility and the agarose gel concentration, in these experiments the percentage agarose (w/v) was chosen to maximalyy seperate the linear DNA fragments of interest (Table 6.1, Sambrook *et al.*, 1989). The DNA concentration was estimated by electrophoresing a small aliquot of the isolated DNA fragment on an agarose gel and comparing its band intensity to the most similarly sized bond of the λHind III molecular weight marker.

2.3.9 Agarose Gel Centrifugation Method

DNA fragment(s) to be isolated were separated by size using electrophoresis through an agarose gel of the appropriate concentration. SeaKem^{R} Le Agarose was used, since other agarose preparations may contain inhibitors of DNA ligase. TBE (1X) buffer was used for electrophoresis and ethidium bromide at a concentration of 0.25 $\mu g ml^{-1}$ was incorporated both into the agarose gel and electrophoresis buffer.

DNA fragment(s) were isolated from the smallest volume of gel slice and centrifuged through filter paper according to the methods of Weichenham *et al.* (1991). In this method, a square 1 cm x 1 cm piece of Whatman 3 (Whatman Ltd, England) filter paper was carefully wrapped around the blunt-end of a micropipette tip to form a 'beaker' that was carefully fitted tightly into a 0.75 ml eppendorf tube that had been punctured at the bottom. The agarose slice containing the DNA was placed into this 'beaker'. This eppendorf tube was mounted into a second (not punctured) 1.5 ml eppendorf tube and this assembly was centrifuged for 1 minute at maximum speed in a microfuge (Eppendorf; Model 5414S). This TBE-containing solution centrifuged from the agarose gel was extracted once with a phenol:chloroform:isoamyl alcohol (25:24:1) solution and precipitated using ethanol (Sambrook *et al.*, 1989).

2.3.10 Dephosphorylation

Calf intestinal alkaline phosphatase (CIAP; Boehringer Mannheim) was used to remove the 5'-phosphate groups from the blunt-ends of the linearized vector DNA, to prevent them from self-ligating.

A protocol supplied by Boehringer Mannheim (1989) was used according to the manufacturers specifications for blunt-ended DNA. Although this enzyme was selected over bacterial alkaline phosphatase (BAP) because of its greater sensitivity to inactivation by heat and detergents, it was nevertheless removed after dephosphorylation by extracting the DNA solution with 1 volume of a phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1) solution and then precipitating the DNA with ethanol (Sambrook *et al.*, 1989).

2.3.11 Conversion of 5-Protruding Ends to Blunt-Ends

The 5'-3' polymerase activity of the Klenow fragment from *E. coli* DNA polymerase I (Megaprime DNA labelling kit (RPL); RPN 1607, Amersham), was used to fill the recessed 3'-termini of DNA fragments created by restriction endonuclease digestion. The protocol used was an adaptation of the methods by Sambrook *et al.* (1989). Up to 1 µg of DNA was incubated with 1 unit of DNA Polymerase I Klenow fragment, 0.5 mM of each of the 4 dNTPs in 25 µl of 1X restriction endonuclease buffer L (1 mM Tris-HCL; 1 mM MgCl; 0.1 mM dithioerythritol; Boehringer Mannheim). This was incubated for 30 to 45 minutes at 22 °C and the DNA Polymerase I Klenow fragment was deactivated by heating to 75 °C for 10 minutes. Because the recessed 3'-termini of the DNA fragments to be filled were first purified on an agarose gel, buffer L was the buffer of choice because of the requirement DNA polymerase I Klenow fragment has for magnesium ions as a cofactor.

Because bacteriophage T4 DNA ligase is not significantly inhibited by the presence of the unincorporated dNTPs, they were not removed from the DNA solution before ligation.

2.3.12 Ligations

Before ligation, all other enzyme activities were eliminated. The DNA fragments were deproteinized with 1 volume of a phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1) solution and the DNA precipitated by ethanol, according to Sambrook *et al.* (1989) to remove any salt-containing buffer.

Ligation reactions were incubated with 1 unit (5 Weiss units; Weiss *et al.*, 1968) of bacteriophage T4 DNA ligase in 10 µl of the ligation buffer for 20 hours at 22 °C

(Bercovich *et al.,* 1992), using a vector:insert ratio of 1 and a calculated *j/i* ratio of 2 (Appendix 1). After incubation, an aliquot of the ligation mixture was electrophoresed on a 0.8% agarose gel to monitor the amount of closed circular DNA formed in the ligation reaction before its transformation into 'competent' *E. coli* DH5α cells. Because ATP is degraded with multiple freeze-thaw cycles, the buffer for the bacteriophage T4 DNA ligase was stored in 20 µl aliquots at -20 °C and thawed only twice.

Parallel ligation and transformation reactions were set up as follows:-

Control #1. A transformation reaction which received no DNA to check for plasmid contamination in either the 'competent' cells and/or transformation buffer. It was also a check that the LB agar plates contained the correct antibiotic.

Control #2. Plasmid vector isolated using the alkaline lysis miniprep procedure was used to assess the transformation efficiency of 'competent' *E. coli* DH5α cells. Plasmid DNA isolated using a miniprep procedure is generally contaminated with phenol protein etc, which lowers the efficiency of transformation.

Control #3. Linearized vector containing blunt-ended termini was used as a ligation control without the addition of T4 DNA ligase to indicate the efficiency of restriction digestion. The presence of colonies was indicative of a background of uncleaved vector DNA. Although linearized DNA can be transformed into *E. coli* 70% as efficiently as circular DNA (Hanahan, 1983), it is usually degraded by the host enzymes (Brown, 1990).

Control #4. Linearized vector containing blunt ended termini was self-ligated to assess the efficiency of the ligation reaction.

Control #5. Linearized vector containing blunt ended termini was dephosphorylated and then religated, to assess the efficiency of the dephosphorylation reaction.

Control #6. Plasmid pPB74 provided by Damak (1991), containing a DNA insert cloned into the Eco RI site of pUC19 vector was cleaved with Eco NI, which cleaves 4 times within the insert. The linearized fragment containing the pUC19 vector had a single 5'-overhang on either end which cannot self-ligate or ligate to another molecule of the same DNA species unless it was blunt-ended. This fragment was DEAE-purified and used as a control for the 5'-3' polymerase activity of the DNA polymerase I Klenow fragment.

2.3.13 Preparation of 'Competent' *E. coli* Cells

A stock of *E. coli* strain DH5α cells devoid of plasmid were supplied by Bethesda Research Laboratories. They were harvested during the mid-log phase of their growth ($O.D_{590}$ = 0.375) and made 'competent' for DNA uptake by incubating at low temperatures in the presence of $CaCl_2$ (Cohen *et al.*, 1972, modified by Sambrook *et al.*, 1989). They were stored at -100 °C in 200 µl aliquots to avoid repeated thawing. These cells had a transformation efficiency of 6.8 x 10^6 when using a pure preparation of pSK Bluescript.

2.3.14 Transformation of DNA Into 'Competent' *E. coli* Cells

The procedure used to transform the *E. coli* cells with the ligated DNA samples was adapted from the protocol supplied by Bethesda Research Laboratory. Competent *E. coli* DH5α cells were removed from -100 °C freezer and thawed slowly on ice. When just thawed, the cells were mixed gently and 100 µl (1.25 x 10^8 to 1.75 x 10^8 cells) were aliquoted into each of the required number of chilled (Falcon^R 2059) polypropylene tubes using pre-cooled sterile pipette tips. Five microlitres of each ligation mixture was added by moving a pre-cooled sterile pipette tip through the 'competent' cells while dispensing. These were then gently shaken for approximately 5 seconds immediately after addition of the DNA and incubated once for 30 minutes. The cells were then heat shocked for 45 seconds in a 42 °C waterbath without shaking, to stimulate uptake of the DNA into the 'competent' cells. These were then

placed on ice for 2 minutes, and 0.9 ml of RT SOC, a non-selective media (Hanahan, 1983), was added. The 'competent' cells containing the ligated plasmid were shaken at 225 rpm for 1 hour at 37 °C so that the antibiotic resistance genes on the plasmid were expressed. Under sterile conditions, 50 µl of the cells were spread evenly over LB plates (supplemented with 60 $\mu g ml^{-1}$ of ampicillin), using an L-shaped Pasteur pipette (bent in a Bunsen burner) and allowed to dry at room temperature. The plates were then inverted and incubated at 37 °C for 20 hours to allow colonies of transformed cells to grow to a detectable size. Colonies that carried the desired recombinant plasmid were identified by restriction analysis of miniprep plasmid DNA.

The efficiency of transformation was expressed as colonies formed per microgram of vector DNA (Hanahan, 1983; 1991). From this, efficiencies of ligation were calculated.

1. The efficiency of self-ligation of vector (control #4) was calculated (Draper *et al.*, 1988) as follows:-

$$\frac{CONTROL\ No.\ 4\ (colonies\ \mu g^{-1}\ vector)}{CONTROL\ No.\ 2\ (colonies\ \mu g^{-1}\ vector)} \times \frac{100}{1} = \%\ efficiency\ of\ ligation$$

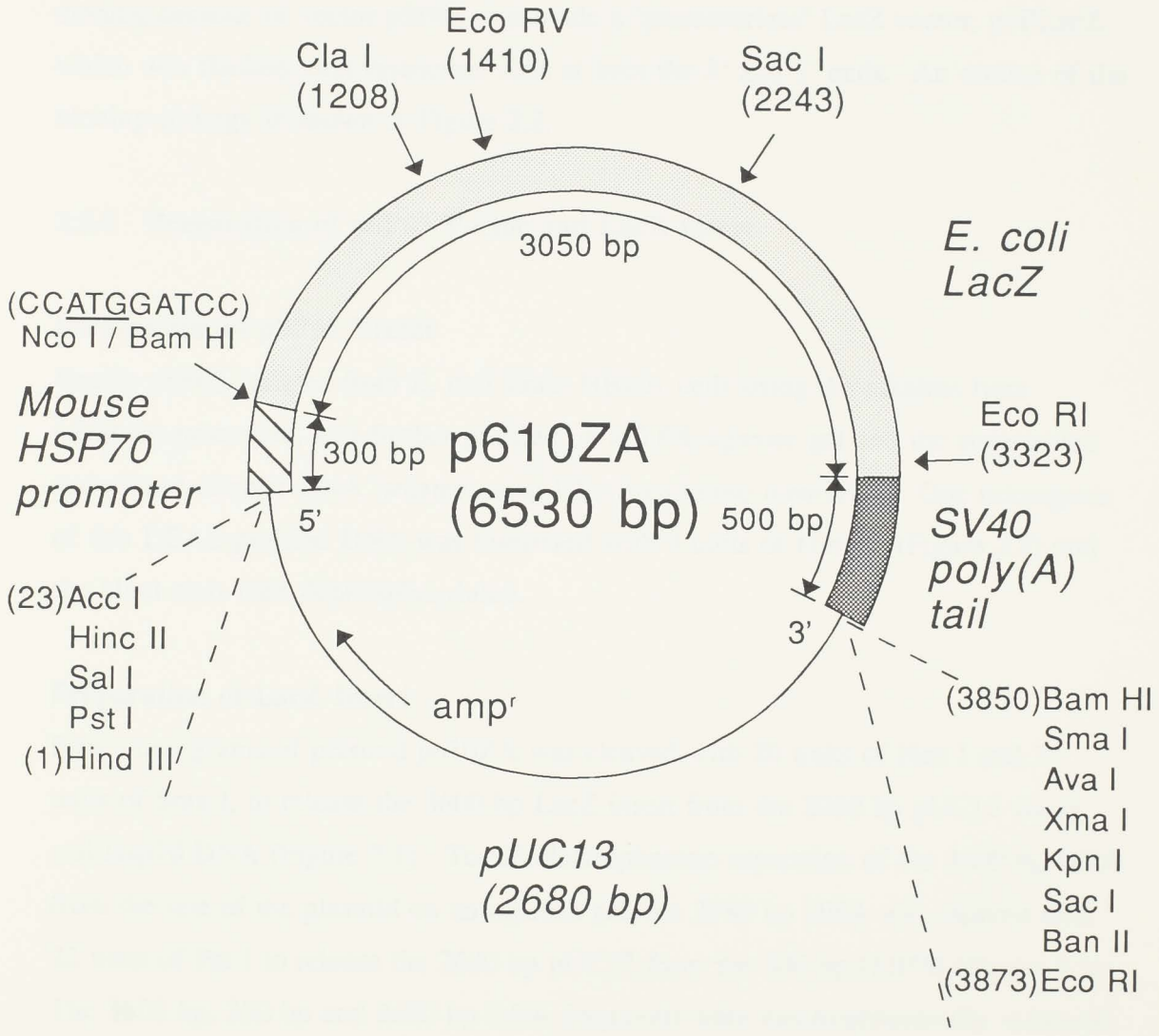
2. Inhibition of vector self-ligation by dephosphorylation (control #5) was calculated (Draper *et al.*, 1988) as follows:-

$$\frac{CONTROL\ No.\ 4\ (colonies\ \mu g^{-1}\ vector) - CONTROL\ No.\ 5\ (colonies\ \mu g^{-1}\ vector)}{CONTROL\ No.\ 4\ (colonies\ \mu g^{-1}\ vector)} \times \frac{100}{1}$$

2.4 Introduction

These cloning experiments were carried out to produce a mammalian expression vector. The first step involved the construction of a promoterless *E. coli* LacZ cloning vector, pSPLacZ, where the LacZ gene was flanked by the 5'-Kozak translation initiation sequence and a 3'-Sv40 polyadenylation signal.

Figure 2.1 Restriction Map of Plasmid p610ZA.



Fragments of 3.3 kb (-3254/+9) and 0.4 kb (-385/+9) from the 5'-flanking uteroglobin DNA were then inserted 5' to the LacZ reporter gene in this pSPLacZ construct to give plasmids pUG3.3LacZ and pUG400LacZ.

2.5 Stage I: Cloning of *E. coli* LacZ into pSP65 Vector

The 3600 bp *E. coli* LacZ fragment from p610ZA was subcloned into the multiple cloning cassette of vector pSP65 to provide a 'promoterless' LacZ vector, pSPLacZ, which was flanked with restriction sites at both the 5' and 3' ends. An outline of the cloning strategy is shown in Figure 2.2.

2.5.1 Preparation of pSP65 Vector and LacZ Insert

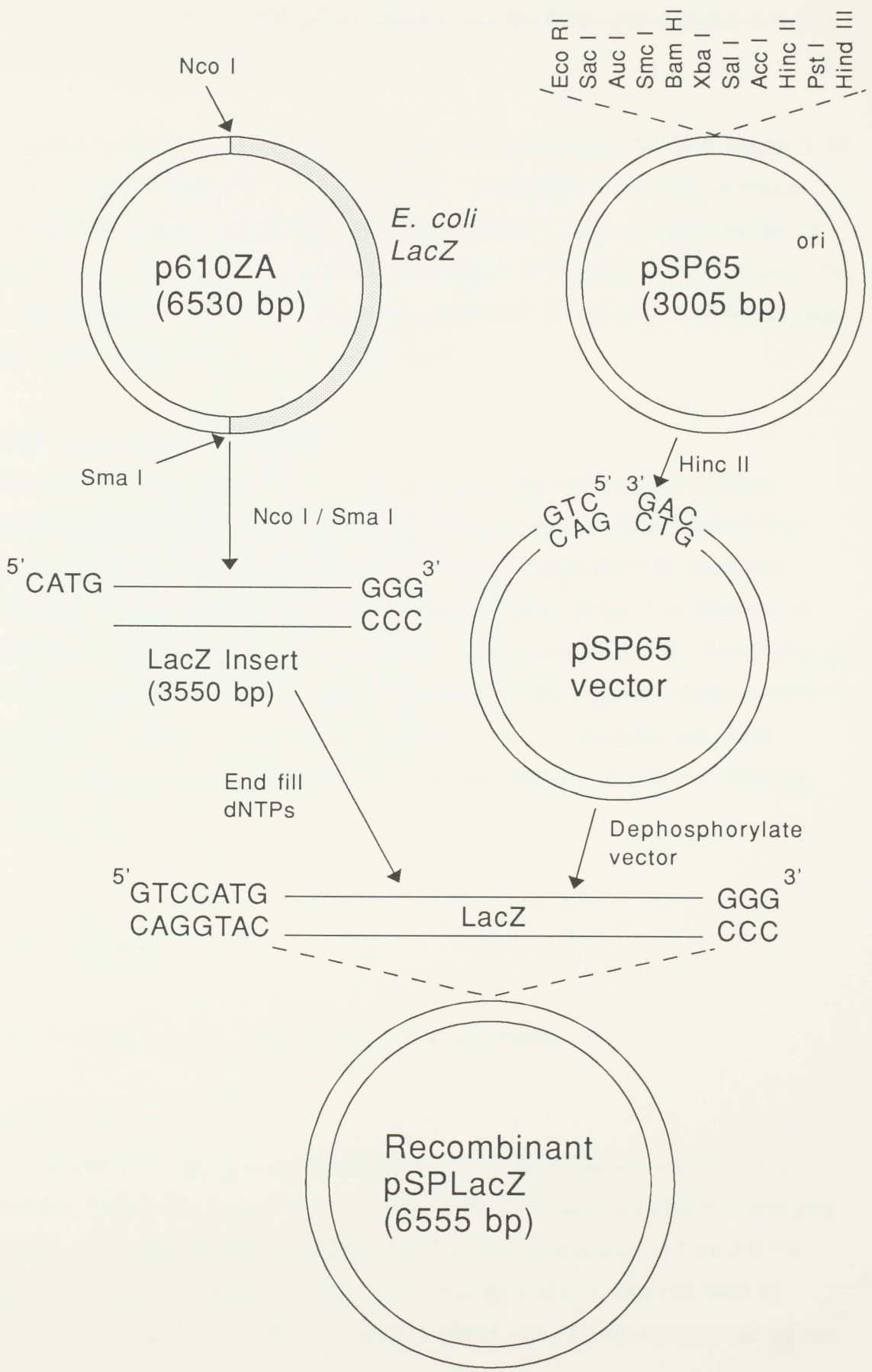
Preparation of pSP65 Vector

Vector pSP65 isolated from *E. coli* strain HB101 cells using the alkaline lysis miniprep procedure, was further purified on a 0.8% agarose gel and the supercoiled and closed-circular DNA isolated using DEAE-cellulose membrane. One microgram of this DEAE-purified DNA was linearized with 4 units of Hinc II (Figure 2.2) and the blunt-ends then dephosphorylated.

Preparation of LacZ Insert

Fifty micrograms of plasmid p610ZA was cleaved with 20 units of Nco I and 24 units of Sma I, to release the 3600 bp LacZ insert from the 2980 bp pUC13 vector and Hsp70 DNA (Figure 2.1). To aid electrophoretic separation of the 3600 bp DNA from the rest of the plasmid on an agarose gel, the 2980 bp DNA was cleaved with 22 units of Pst I to release the 2680 bp pUC13 from the 300 bp HSP70 (Figure 2.1). The 3600 bp, 300 bp and 2680 bp DNA fragments were electrophoretically separated on a 0.8% agarose gel and the LacZ insert was isolated using DEAE-cellulose membrane. The 5'-CATG-3' sticky end created by Nco I was converted to a blunt-end using the Klenow fragment from *E. coli* DNA polymerase I (Section 2.3.11).

Figure 2.2 Construction of Recombinant pSPLacZ.



2.5.2 Ligation of LacZ into pSP65

The 3600 bp LacZ insert fragment was blunt end ligated into the Hinc II restriction site of vector pSP65. Parallel control ligation and transformation reactions were set up according to Section 2.3.12.

Ligation reactions were incubated with 1 unit of bacteriophage T4 DNA Ligase in 10 µl of 1X ligation buffer for 20 hours at 22 °C. To increase the amount of closed circular DNA formed in the ligation reaction, another 0.5 unit of bacteriophage T4 DNA ligase was added and incubation continued at 22 °C for a further 17 hours. The resulting plasmids were transformed into 'competent' *E. coli* strain DH5α cells and the transformation efficiencies were calculated.

2.5.3 Analysis of Recombinants

Plasmid DNA was isolated from *E. coli* cells using the alkaline lysis miniprep procedure and plasmids containing inserted foreign DNA was distinguished from vector molecules recircularized without insertion of foreign DNA by restriction mapping. Correct orientation of the 3600 bp LacZ insert in the 5'-3' direction in pSP65 was established by cleaving this DNA with 5 units of Eco RI. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave DNA fragment lengths of 3084 bp and 3505 bp if the LacZ insert was orientated in the 5'-3' direction and DNA fragment lengths of 500 bp and 6055 bp if it was orientated in the 3'-5' direction (Figure 2.3).

2.6 Results

2.6.1 Preparation of pSP65 Vector and LacZ Insert

pSP65 Vector

The alkaline lysis miniprep for pSP65 yielded 1.13 µg of plasmid per ml of LB medium; with an A_{260}/A_{280} ratio of 1.4. To verify its identity, an aliquot of miniprep DNA was cleaved with Rsa I. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave the expected DNA fragment lengths of 676, 444 and 1883 bp (Figure 2.2). There was a 70% recovery of pSP65 using DEAE-cellulose membrane.

LacZ Insert

Plasmid p610ZA purified on a caesium chloride gradient, had an A_{260}/A_{280} purity ratio of 1.44. The total yield of the 3600 bp LacZ fragment isolated from p610ZA using DEAE-cellulose membrane was 8.9 µg, a recovery of 33%. An aliquot of this DNA electrophoresed in a 1.2% agarose gel indicated that only the 3600 bp LacZ fragment was purified.

2.6.2 Ligation of LacZ into pSP65

An aliquot of the ligation reaction electrophoresed in a 0.8% agarose gel demonstrated that closed circular DNA had formed. The presence of ligated bands for pPB74 (control #6) demonstrated that the Klenow fragment had blunt-ended the single-base 5'-overhang. The presence of only one linear band for the dephosphorylated pSP65 vector (control #5) indicated that the dephosphorylation reaction had removed most of the 5'-phosphates from the vector DNA.

Table 2.1 Ligation and Transformation Efficiencies of LacZ subcloned into pSP65.

Control #	Vector (ng)	Insert (ng)	Amt. DNA Transf. (ng)	# Colonies	(per µg)
(1) No DNA	-	-	-	0	(0)
(2) pSP65	60	-	1.5	1000	(6.7×10^5)
(3) Linearized pSP65 Vector	50	-	1.25	22	(1.76×10^4)
(4) Religated pSP65 Vector	50	-	1.25	400	(3.2×10^5)
(5) Dephos. pSP65 Vector	30	-	0.75	26	(3.4×10^4)
Dephos. pSP65 Vector & Insert	134	164	7.45	20	(4.9×10^3)
# Recombinants				2/12	(4.47×10^2)

2.6.3 Transformation of Strain DH5α

Absence of colonies in the control transformation reaction (control #1) indicated that there was no contaminating ampicillin resistant DNA present in either the 'competent' cells and/or the buffers used for their transformation.

The transformation efficiency for the minipreparation of pSP65 (control #2) was good at 6.7×10^5 colonies μg^{-1} of DNA. The background rate of transformation from pSP65 linearized (control #3), was high at 1.76×10^4 colonies μg^{-1} DNA; indicative of a high background of circular plasmid DNA that was uncleaved.

The efficiency of pSP65 religating to itself (control #4) was 47.8%. However because of the high background of circular plasmid DNA present in control #3; artificially inflating the transformation and religation efficiency of pSP65 vector, the true transformation efficiency was obtained for control #4 as follows:

CONTROL No. 4 (3.2×10^5) - *CONTROL No.* 3 (1.76×10^4) = 3.2×10^5 *colonies* μg^{-1} *vector.*

The effective efficiency of religation for pSP65 was therefore 45.1% when corrected for this high background of circular vector.

The dephosphorylation reaction (control #5) resulted in a transformation efficiency of 3.4×10^4 colonies μg^{-1} vector compared with 3.2×10^5 colonies μg^{-1} vector (control #4) prior to dephosphorylation. These figures would however include the background uncleaved DNA (control #3), artificially inflating these figures. The effective transformation efficiency for the dephosphorylation reaction (control #5) was therefore calculated as follows:

CONTROL No. 5 (3.4×10^4) - *CONTROL No.* 3 (1.76×10^4) = 1.64×10^4 *colonies* μg^{-1} *vecto*

Using these figures, it was calculated that there was a 94.6% inhibition of self-ligation by dephosphorylation.

Figure 2.3 **Restriction Map of pSPLacZ.**

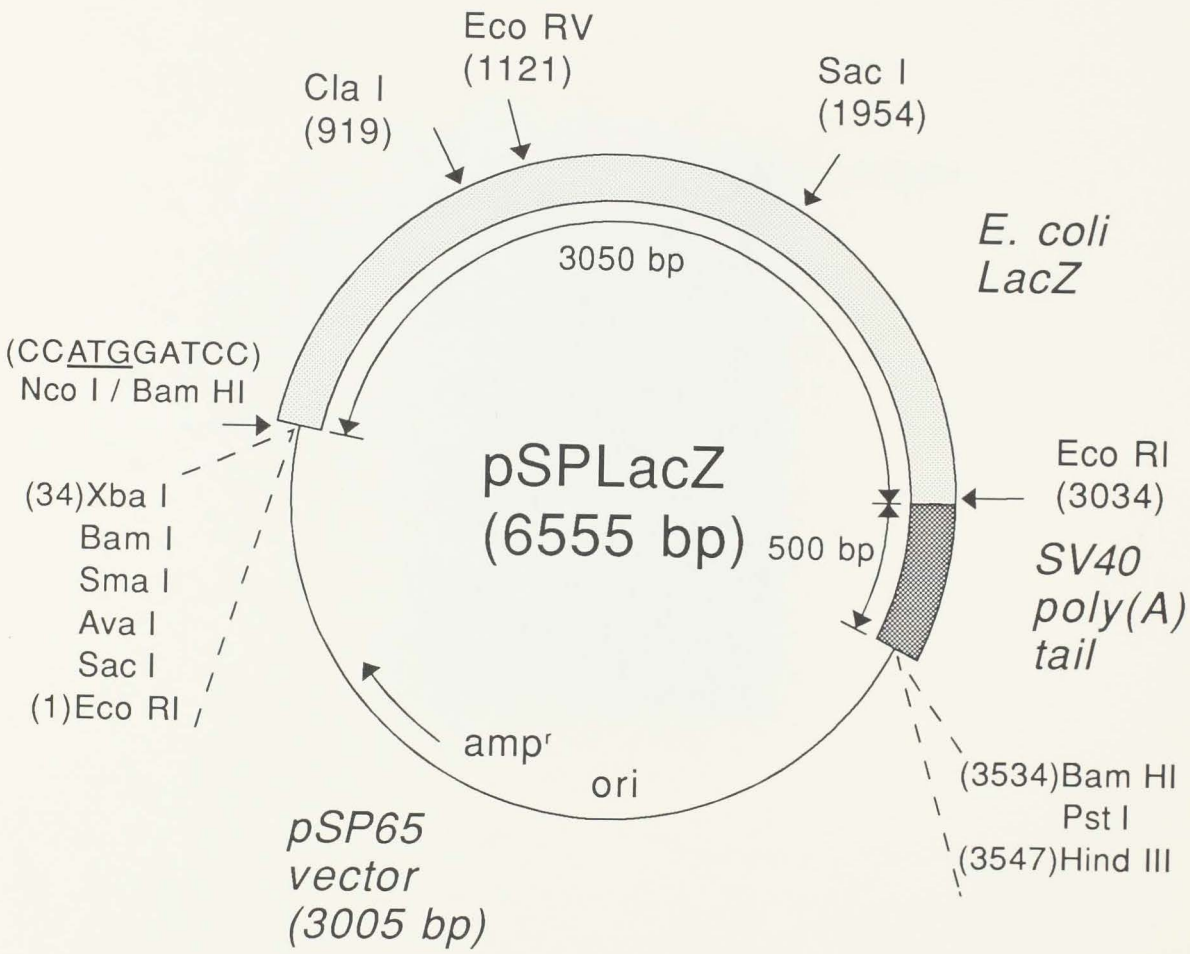


Figure 2.4 Restriction digest of pSPLacZ.

Lane 1: λHind III molecular weight marker

Lane 2: pSPLacZ cleaved with Nco I

Lane 3: pSPLacZ cleaved with Pst I / Cla I

Lane 4: pSPLacZ cleaved with Eco RI

There were 20 colonies for the experimental ligation, representing a transformation efficiency of 4.9 x 10^3 colonies μg^{-1} of LacZ insert. The number of these colonies containing the LacZ insert was 4.47 x 10^2 colonies μg^{-1} of DNA.

2.6.4 Analysis of Recombinants

All 20 colonies from the experimental ligation were selected for miniprep analysis. After 17 hours, 12 of the 20 colonies had grown in the LB medium and a further 4 grew after 24 hours. Plasmid DNA was extracted using alkaline lysis from the 12 colonies which had grown initially.

When cleaved with Eco RI, two transformants contained the LacZ insert and these were both oriented in the desired 5'-3' direction within pSP65. To further confirm that these were the desired recombinants, they were double restricted with 10 units of Cla I and 11 units of Pst I. Confirmation of both recombinants orientated in the 5'-3' direction was provided with DNA fragment lengths of 2628 bp and 3927 bp rather than 5670 bp and 885 bp (3'-5' direction) when electrophoresed on a 0.8% agarose gel.

The desired recombinant was designated pSPLacZ (Figure 2.3; 2.4).

2.7 Stage II: Cloning of UG400 into pSPLacZ

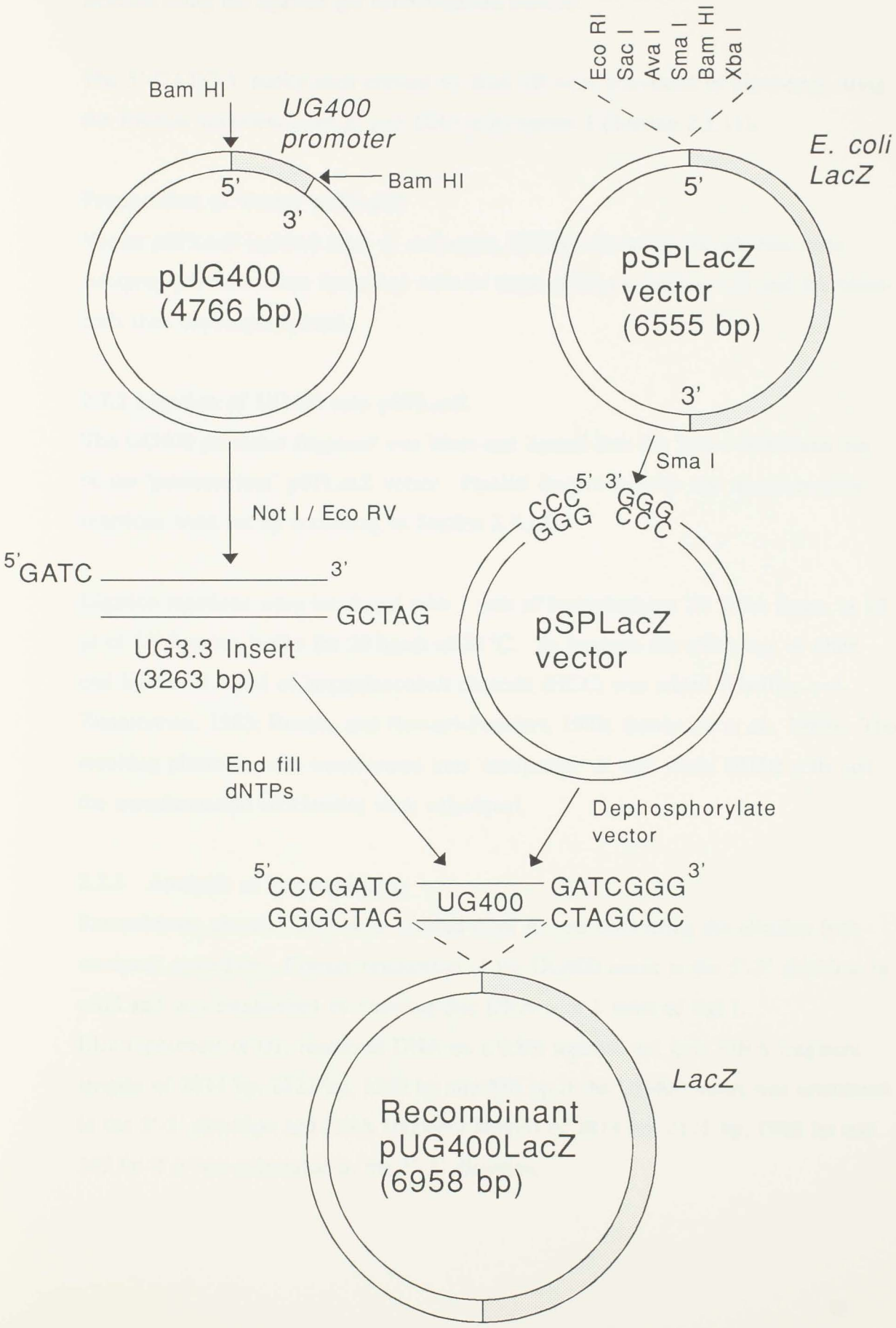
The 403 bp 5'-flanking uteroglobin promoter (UG400) was subcloned into the multiple cloning site of vector pSPLacZ. An outline of the cloning strategy is shown in Figure 2.5.

2.7.1 Preparation of pSPLacZ Vector and UG400 Insert

Preparation of UG400 Insert

Plasmid pUG400 isolated from *E. coli* strain DH5α cells using the alkaline lysis miniprep procedure, was cleaved with 20 units of Bam HI to release the 403 bp uteroglobin insert from the rest of the plasmid (Figure 2.6). These DNA fragments

Figure 2.5 Construction of Recombinant pUG400LacZ.



were electrophoretically separated on a 0.8% agarose gel and the UG400 insert was isolated using the agarose gel centrifugation method.

The 5'-GATC-3' sticky ends created by Bam HI were converted to blunt-ends using the Klenow fragment from *E. coli* DNA polymerase I (Section 2.3.11).

Preparation of Vector pSPLacZ

Vector pSPLacZ isolated from *E. coli* strain DH5α cells using the alkaline lysis miniprep procedure was linearized with 24 units of Sma I (Figure 2.3) and the blunt-ends then dephosphorylated.

2.7.2 Ligation of UG400 into pSPLacZ

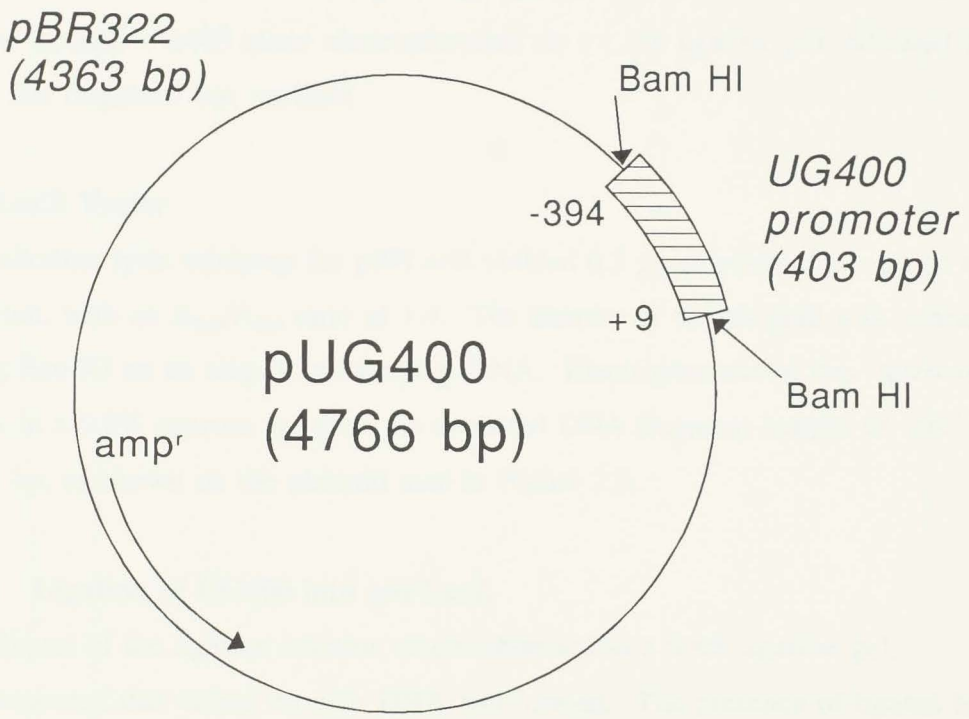
The UG400 promoter fragment was blunt-end ligated into the Sma I restriction site of the 'promoterless' pSPLacZ vector. Parallel control ligation and transformation reactions were set up according to Section 2.3.12.

Ligation reactions were incubated with 1 unit of bacteriophage T4 DNA ligase in 10 µl of 1X ligation buffer for 20 hours at 22 °C. To increase the efficiency of blunt end ligation 1.5 uM of hexaminecobalt chloride (HCC) was added (Pheiffer and Zimmerman, 1983; Rusche and Howard-Flanders, 1988; Sambrook *et al.*, 1989). The resulting plasmids were transformed into 'competent' *E. coli* strain DH5α cells and the transformation efficiencies were calculated.

2.7.3 Analysis of Recombinants

Recombinant plasmid DNA was isolated from *E. coli* cells using the alkaline lysis miniprep procedure. Correct orientation of the UG400 insert in the 5'-3' direction in pSPLacZ was established by cleaving this DNA with 8 units of Bgl I.
Electrophoresis of this restricted DNA on a 0.8% agarose gel gave DNA fragment lengths of 2814 bp, 2121 bp, 1599 bp and 530 bp if the UG400 insert was orientated in the 5'-3' direction and DNA fragment lengths of 2814 bp, 2121 bp, 1966 bp and 163 bp if it was orientated in the 3'-5' direction.

Figure 2.6 Restriction Map of pUG400.



2.8 Results

2.8.1 Preparation of pSPLacZ Vector and UG400 Insert

UG400 Insert

The alkaline lysis miniprep procedure for pUG400 yielded 89 ng of plasmid per ml of LB medium; with an A_{260}/A_{280} ratio of 1.4. To verify its identity, an aliquot of miniprep DNA was cleaved with Bam HI. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave the expected DNA fragment lengths of 4363 bp and 403 bp as shown on the plasmid map in Figure 2.6. There was a 75% recovery of the UG400 insert from pUG400 using the agarose gel centrifugation method. An aliquot of the isolated UG400 insert electrophoresed on a 1.2% agarose gel indicated that only this fragment was purified.

pSPLacZ Vector

The alkaline lysis miniprep for pSPLacZ yielded 0.5 µg of plasmid per ml of LB medium, with an A_{260}/A_{280} ratio of 1.4. The identity of the plasmid was verified using Eco RI on an aliquot of miniprep DNA. Electrophoresis of this 'restricted' DNA in a 0.8% agarose gel gave the expected DNA fragment lengths of 3084 bp and 3505 bp, as shown on the plasmid map in Figure 2.3.

2.8.2 Ligation of UG400 into pSPLacZ

An aliquot of the ligation reaction electrophoresed in a 0.8% agarose gel, demonstrated that closed circular DNA had formed. The presence of ligated bands for pPB74 (control #6) demonstrated that the Klenow fragment had blunt-ended the single-base 5'-overhang.

The presence of only one linear band for the dephosphorylated pSPLacZ vector (control #5), indicated that the dephosphorylation reaction had removed most of the 5'-phosphates from the vector DNA.

Table 2.2 Ligation and Transformation Efficiencies of UG400 Subcloned into pSPLacZ.

Control #	Vector (ng)	Insert (ng)	Amt. DNA Transf. (ng)	# Colonies	(per µg)
(1) No DNA	-	-	-	0	(0)
(2) pSPLacZ	100	-	2.5	600	(2.4×10^5)
(3) Linearized pSPLacZ Vector	70	-	1.75	0	(0)
(4) Religated pSPLacZ Vector	70	-	1.75	0	(0)
(5) Dephos. pSPLacZ Vector	5	-	0.13	2	(1.6×10^4)
Dephos. pSPLacZ Vector & Insert	2800	343	78.6	33	(3.8×10^3)
# Recombinants				1/15	(27.9)

2.8.3 Transformation of Strain DH5α

Absence of colonies in the control transformation reaction (control #1) indicated that there was no contaminating ampicillin resistant DNA present in either the 'competent' cells and/or the buffers used for their transformation.

The transformation efficiency for the minipreparation of pSPLacZ (control #2) was 2.4×10^5 colonies μg^{-1} of vector.

There were no colonies for 'restricted pSPLacZ' (control #3), which indicated there was no contaminating circular plasmid.

The dephosphorylation reaction (control #5) resulted in a transformation efficiency of 1.6×10^4 colonies μg^{-1} of DNA. Because there were no colonies for pSPLacZ self-ligated (control #4), calculation of the efficiency of the dephosphorylation reaction (control #5) was not possible.

There were 33 colonies for the experimental ligation, which represented a transformation efficiency of 3.8×10^3 colonies μg^{-1} of insert UG400. The number of these colonies containing UG400 insert was 27.9 colonies μg^{-1} of DNA.

2.8.4 Analysis of Recombinants

Sixteen of the 33 colonies from the experimental ligation were selected for miniprep analysis. Fifteen out of the 16 colonies had grown after 17 hours in the LB medium. Plasmid DNA was extracted from these 15 colonies using alkaline lysis.

When cleaved with Bgl I, one of the transformants contained the UG400 insert and this was oriented in the 5'-3' direction within pSPLacZ.

The desired recombinant was designated pUG400LacZ (Figure 2.7; 2.8).

Figure 2.7 Restriction Map of pUG400LacZ.

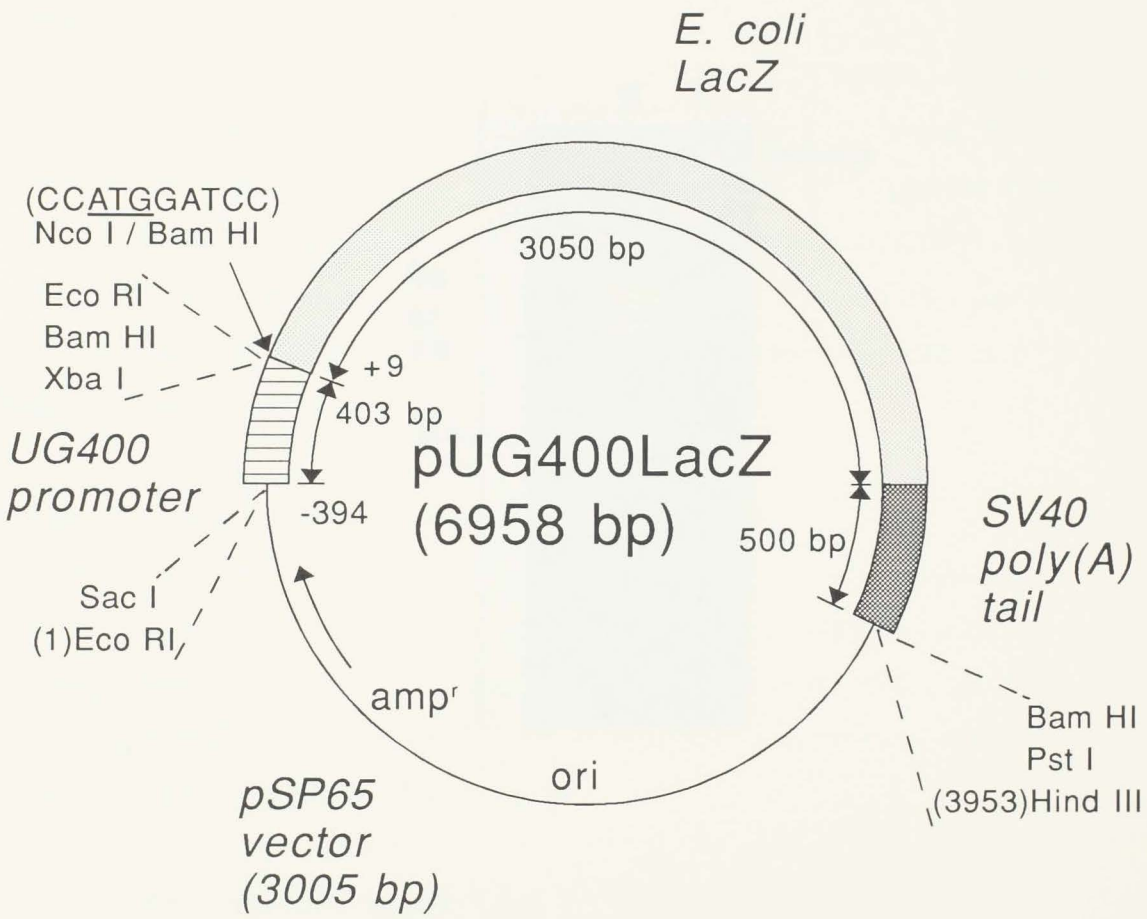
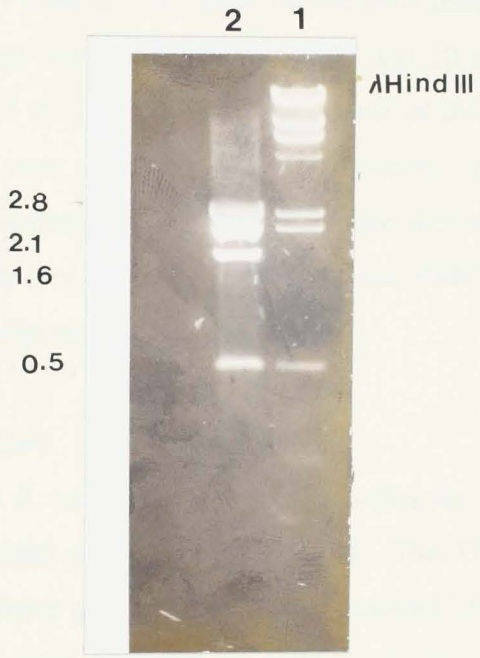


Figure 2.8 Restriction digest of pSPLacZ.



Lane 1: λHind III molecular weight marker

Lane 2: pUG400LacZ cleaved with Bgl I

2.9 Stage III: Cloning of UG3.3 into pSPLacZ

The 3263 bp 5'-flanking uteroglobin promoter (UG3.3) was subcloned into the multiple cloning site of vector pSPLacZ. An outline of the cloning strategy is shown in Figure 2.9.

2.9.1 Preparation of pSPLacZ Vector and UG3.3 Insert

Preparation of UG3.3 Insert

Plasmid pUG3.3 DNA isolated from *E. coli* strain DH5α cells using the alkaline lysis miniprep procedure was cleaved with 10 units of Eco RV and 10 units of Not I, to release the 3263 bp uteroglobin (UG3.3) insert from the rest of the plasmid (Figure 2.10). These DNA fragments were electrophoretically separated on a 0.8% agarose gel and the UG3.3 insert was isolated using DEAE-cellulose membrane. The 5'-CATG-3' sticky end created by Not I was converted to blunt-ends using the Klenow fragment from *E. coli* DNA polymerase I (Section 2.3.11).

Preparation of pSPLacZ Vector

Vector pSPLacZ isolated from *E. coli* strain DH5α cells using the alkaline lysis miniprep procedure was linearized with 24 units of Sma I. This DNA was electrophoresed on a 0.8% agarose gel to separate the linearized DNA from any uncleaved plasmid and then isolated using DEAE-cellulose membrane. This linearized vector was then dephosphorylated.

2.9.2 Ligation of UG3.3 into pSPLacZ

The UG3.3 uteroglobin promoter fragment was blunt end ligated into the Sma I restriction site of the 'promoterless' pSPLacZ vector. Parallel control ligation and transformation reactions were set up according to Section 2.3.12, however control #3 was omitted because the vector was purified from any contaminating uncleaved circular vector. Ligation reactions were incubated with 1 unit of bacteriophage T4 DNA ligase in 10 µl of 1X ligation buffer for 20 hours at 22 °C. Resulting plasmids were transformed into 'competent' *E. coli* strain DH5α cells and the transformation efficiencies were calculated.

Figure 2.9 **Construction of Recombinant pUG3.3LacZ.**

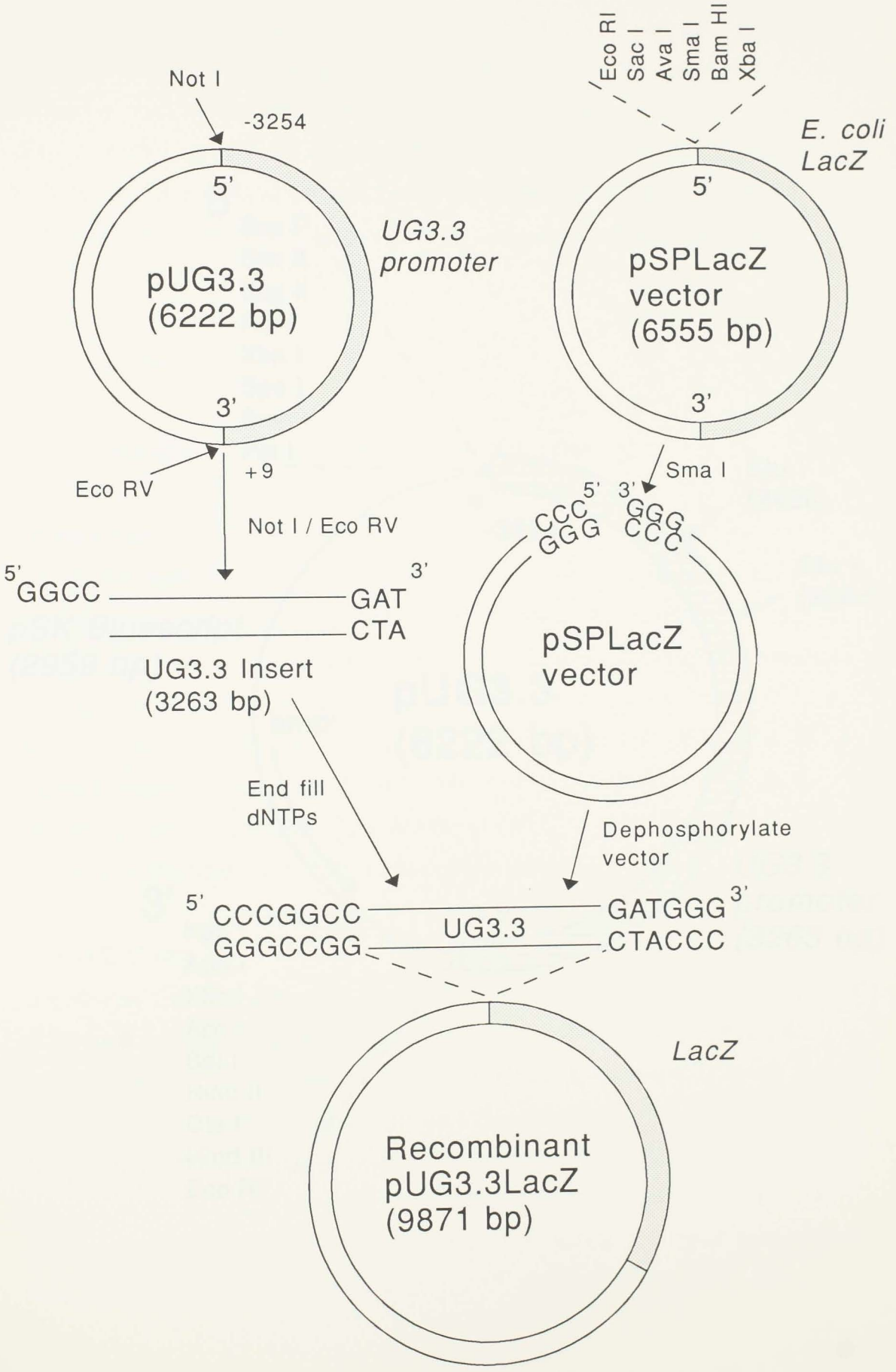
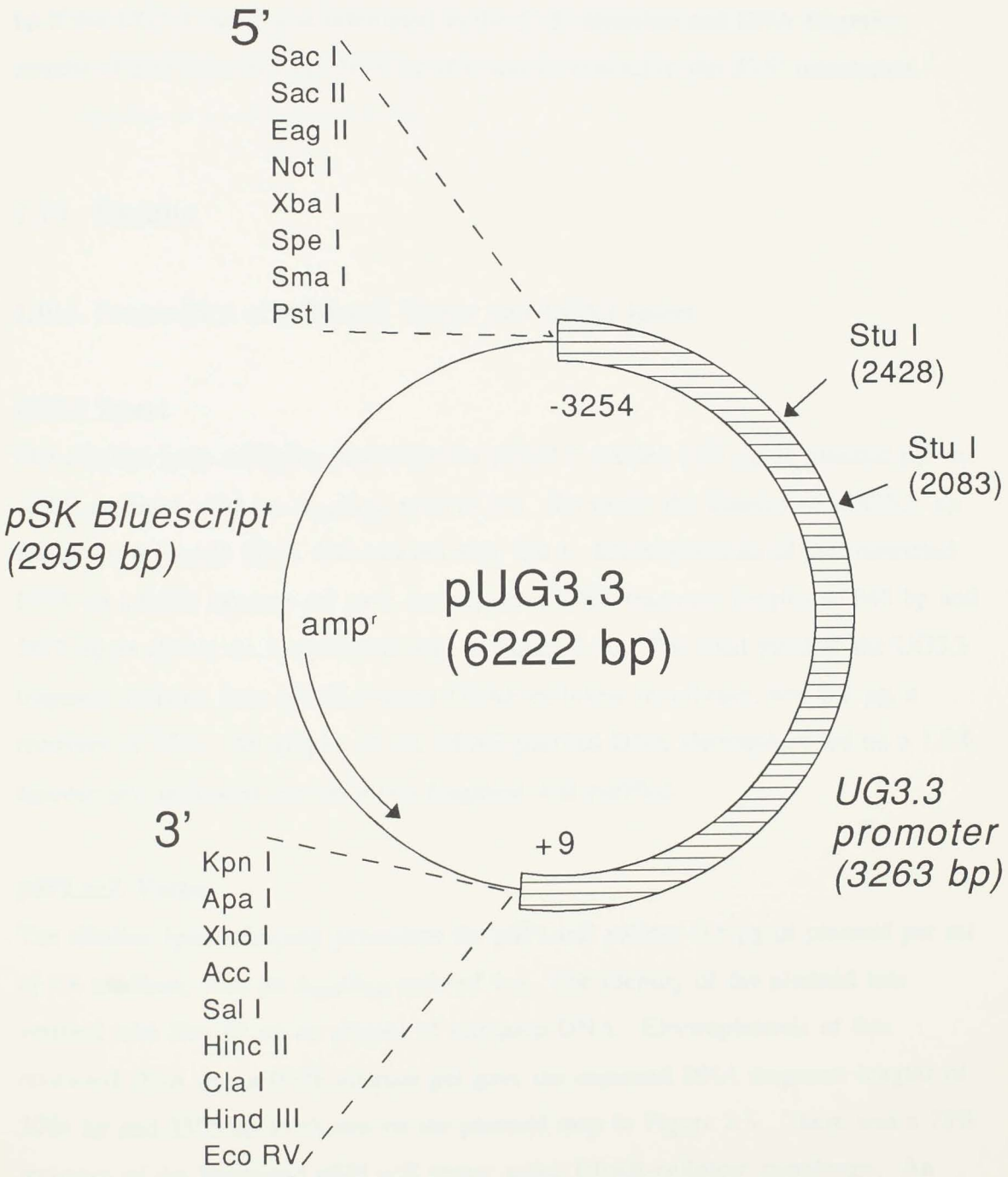


Figure 2.10 Restriction Map of pUG3.3.



2.9.3 Analysis of Recombinants

Recombinant plasmid DNA was isolated from *E. coli* cells using the alkaline lysis miniprep procedure. Correct orientation of the UG3.3 insert in the 5'-3' direction within pSPLacZ was established by cleaving this DNA with 10 units of Eco RV and 10 units of Stu I as a double restriction digest. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave DNA fragment lengths of 345 bp, 3181 bp, 6374 bp if the UG3.3 insert was orientated in the 5'-3' direction and DNA fragment lengths of 345 bp, 1915 bp, 7640 bp if it was orientated in the 3'-5' orientation.

2.10 Results

2.10.1 Preparation of pSPLacZ Vector and UG3.3 Insert

UG3.3 Insert

The alkaline lysis miniprep procedure for pUG3.3 yielded 1.65 µg of plasmid per ml of LB medium; with an A_{260}/A_{280} ratio of 1.4. To verify the identity of pUG3.3, an aliquot of miniprep DNA was cleaved with Stu I. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave the expected DNA fragment lengths of 345 bp and 5877 bp as shown on the plasmid map in Figure 2.10. The total yield of the UG3.3 fragment isolated from pUG3.3 using DEAE-cellulose membrane, was 0.2 µg, a recovery of 75%. An aliquot of the DEAE-purified DNA electrophoresed on a 1.2% agarose gel, indicated that only this fragment was purified.

pSPLacZ Vector

The alkaline lysis miniprep procedure for pSPLacZ yielded 0.5 µg of plasmid per ml of LB medium; with an A_{260}/A_{280} ratio of 1.4. The identity of the plasmid was verified with Eco RI on an aliquot of miniprep DNA. Electrophoresis of this restricted DNA on an 0.8% agarose gel gave the expected DNA fragment lengths of 3084 bp and 3505 bp as shown on the plasmid map in Figure 2.3. There was a 75% recovery of the linearized pSPLacZ vector using DEAE-cellulose membrane. An aliquot of this DNA was electrophoresed on a 1.2% agarose gel, which indicated that there were no DNA contaminants present.

2.10.2 Ligation of UG3.3 into pSPLacZ

An aliquot of the ligation reaction electrophoresed on a 0.8% agarose gel demonstrated that closed circular DNA had formed. The presence of ligated DNA for pPB74 (control #6) demonstrated that the Klenow fragment had blunt-ended the single-base 5'-overhang.

The presence of only one linear band for the dephosphorylated pSPLacZ vector (control #5) indicated that the dephosphorylation reaction had removed most of the 5'-phosphates from the vector DNA.

Table 2.3 Ligation and Transformation Efficiencies of UG3.3 Subcloned into pSPLacZ.

Control #	Vector (ng)	Insert (ng)	Amt. DNA Transf. (ng)	# Colonies	(per µg)
(1) No DNA	-	-	-	0	(0)
(2) pSPLacZ	5	-	1.13	52	(4.2×10^5)
(3) Linearized pSPLacZ Vector	50	-	1.25	22	(1.76×10^4)
(4) Religated pSPLacZ Vector	150	-	3.75	-	-
(5) Dephos. pSPLacZ Vector	150	-	3.75	118	(3.1×10^4)
Dephos. pSPLacZ Vector & Insert	350	170	13	144	(3.4×10^4)
# Recombinants				1/19	(5.77×10^2)

2.10.3 Transformation of Strain DH5α

Absence of colonies in the control transformation reaction (control #1) indicated that there was no contaminating ampicillin resistant DNA present in either the 'competent' cells and/or the buffers used for their transformation.

The transformation efficiency for the minipreparation of pSPLacZ (control #2) was 4.2×10^5 colonies μg^{-1} of vector. There were no colonies for pSPLacZ self-ligated (control #4). Because of this the efficiency of dephosphorylation was unable to be calculated. The dephosphorylation reaction (control #5) resulted in a transformation efficiency of 3.1×10^4 colonies μg^{-1} of vector.

There were 144 colonies for the experimental ligation, which represented a transformation efficiency of 3.4×10^4 colonies μg^{-1} of UG3.3 insert. The number of colonies containing UG3.3 insert was 5.77×10^2 colonies μg^{-1} of DNA.

2.10.4 Analysis of Recombinants

Nineteen out of the 144 colonies from the experimental ligation were selected for miniprep analysis. All 19 colonies grew after 17 hours in LB media and plasmid DNA was extracted from these using alkaline lysis.

When cleaved with Stu I and Eco RV, one of the transformants contained the UG3.3 insert and this was oriented in the 5'-3' direction within pSPLacZ. To further confirm that this was the desired recombinant, it was cleaved with 12 units of Sma I. Confirmation that this recombinant was orientated in the 5'-3' direction was provided with DNA fragment lengths of 2700 bp and 7200 bp rather than 9300 bp and 600 bp (3'-5' orientation) when electrophoresed on a 0.8% agarose gel.

This recombinant was designated pUG3.3LacZ (Figure 2.11; 2.12).

Figure 2.11 Restriction Map of Recombinant pUG3.3LacZ.

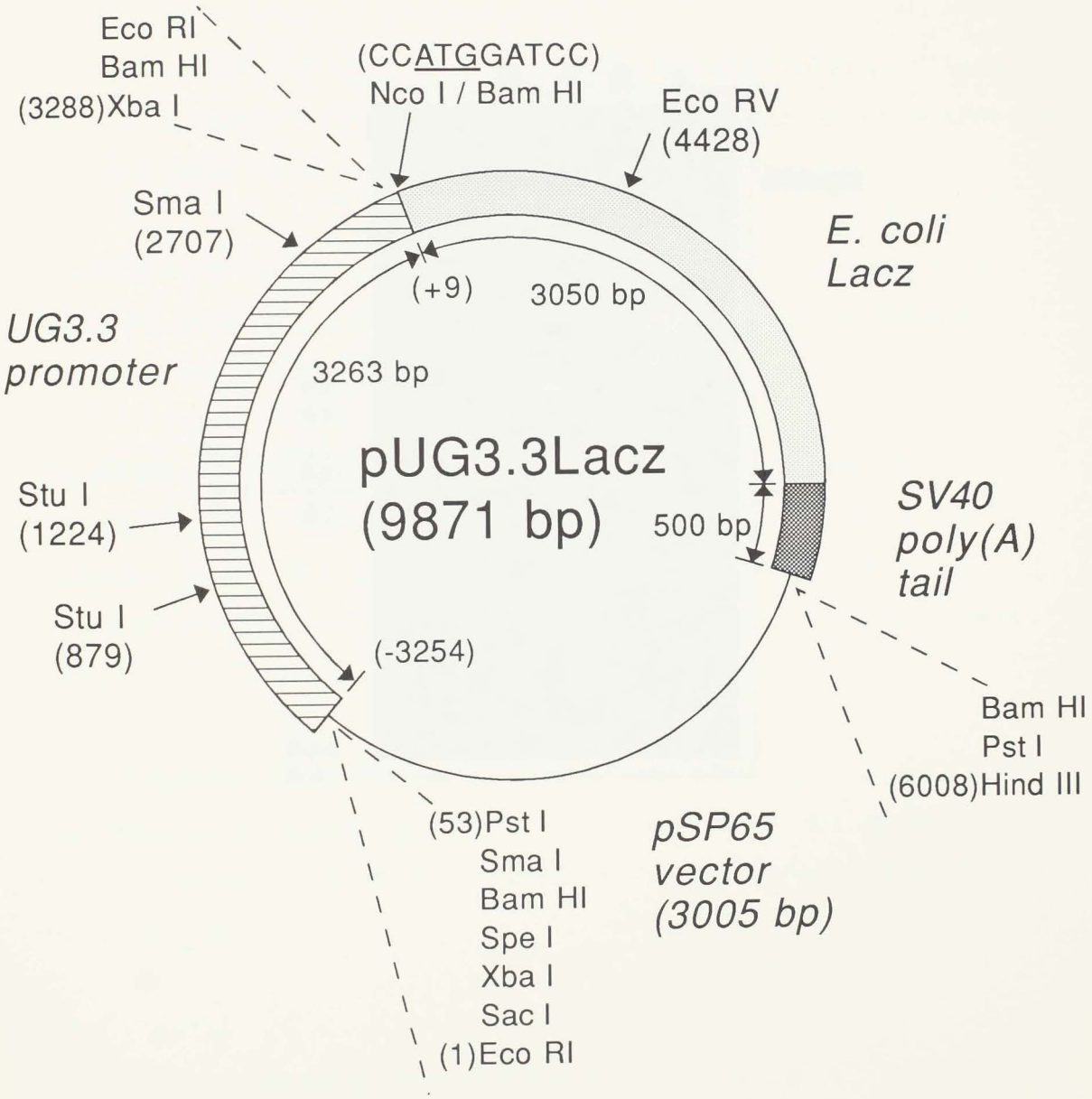
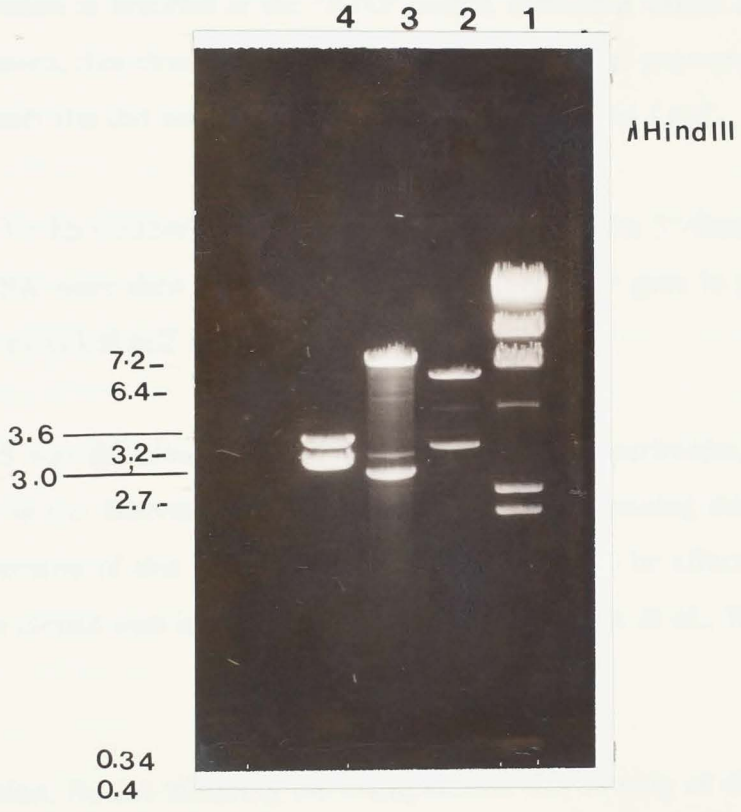


Figure 2.12 Restriction map of pUG3.3LacZ



Lane 1: λHind III molecular weight marker.

Lane 2: pUG3.3LacZ cleaved with Eco RV / Stu I

Lane 3: pUG3.3LacZ cleaved with Sma I

Lane 4: pUG3.3LacZ cleaved with Bam HI

2.11 Discussion

In these experiments, DNA was manipulated to produce an *E.coli* LacZ cloning vector, pSPLacZ. This vector contained the promoterless *E. coli* LacZ structural gene cloned into the multiple cloning site of *E. coli*-based plasmid pSP65. The LacZ structural gene of this pSPLacZ vector was flanked with unique restriction sites Xba I and Sma I at the 5' end and Pst I and Hind III at the 3' end.

Because translation is initiated at the 'ATG' codon, contained within the 5' Kozak initiation sequence, this cloning vector had the advantage that promoters containing a transcription start site did not have to be fused in frame to the LacZ.

Fragments of 3.3 kb (-3254/+9) and 0.4 kb (-385/+9) from the 5'-flanking uteroglobin DNA were then inserted 5' to this LacZ reporter gene in pSPLacZ to give plasmids pUG3.3LacZ and pUG400LacZ respectively.

Plasmid pSP65 was the cloning vector of choice in these experiments. It was not only available in our laboratory but was one of the few containing minimal LacZ sequences. Because of this plasmid stability was unlikely to be affected when the *E. coli* LacZ was cloned into it (Raleigh *et al.*, 1989; Sambrook *et al.*, 1989; Hickford, 1991).

In this discussion, factors affecting the manipulation and cloning of the DNA in these experiments are considered separately. It is acknowledged that this separation is artificial since the overall effect of these factors' combine to affect the outcome of a cloning experiment.

2.11.1 Ligation

Ligation involves the formation of two phosphodiester bonds between the 5'-phosphate groups of one DNA fragment to the 3'-hydroxyl groups of another (Struhl, 1987). Because the restriction sites of the vector DNA were incompatible with the insert DNA in these experiments, DNA fragments were subcloned into their plasmid vectors using blunt-end ligations.

In theory, sticky-end ligation involving complementary DNA termini are a much more efficient reaction than ligation involving blunt-ends. This is because DNA 'sticky-ends' can form transient base-pairs with one another by hydrogen bonding, thereby increasing the length of time they are in contact with one another. Because DNA fragments carrying blunt-ended termini must be cloned in linearized plasmid vectors carrying compatible ends, DNA termini with 5'-sticky ends must be completely filled with dNTPs to produce blunt-ended DNA. These additional reactions further lower the efficiency of blunt-end ligation so that its success, depends on a number of parameters as discussed separately below.

DNA Preparation

Plasmid DNA used for cloning was isolated from E.coli cells on a 'miniprep' scale, using alkaline lysis. This procedure was chosen because being fast and reproducible, it provided several micrograms of plasmid DNA that were readily cleaved with restriction enzymes (Sambrook *et al.*, 1989). Plasmid DNA isolated this way however are frequently contaminated with protein, RNA and chemicals that were used in their isolation (eg. phenol, ethanol etc), all of which significantly lower the purity of the DNA.

In these experiments the A_{260}/A_{280} ratios of the plasmid DNA, isolated using alkaline lysis were 1.4, significantly lower than the ratio of 1.8, cited for pure DNA (Sambrook *et al.*, 1989). The A_{260}/A_{280} ratios obtained in these experiments were similar to others obtained in our laboratory, and were acceptable for cloning purposes.

Because plasmid DNA isolated by the alkaline lysis miniprep method was contaminated with protein and phenol which absorb ultraviolet irradiation, DNA concentration could not be accurately quantified on the spectrophotometer. Instead the amount of DNA was estimated by electrophoresis on an agarose gel. The ultraviolet induced-fluorescence, emitted by the ethidium bromide molecules intercalated into the DNA, was compared with a known quantity of the λHind III molecular weight marker. This was proportional to the total mass of the DNA (Sambrook *et al.*, 1989).

The desired DNA fragments were further purified by agarose gel electrophoresis and isolated using DEAE-cellulose membrane. Gel purifying these DNA fragments for ligation enabled crude miniprep DNA to be used as the initial starting material, instead of DNA purified on a caesium chloride density gradient (Struhl, 1987). DEAE-cellulose membrane was the method of choice for the isolation of DNA fragments. DNA fragments isolated using this method has routinely been used in blunt-end ligation (Dretzen *et al.*, 1981). Yields of DNA recovered using this method ranged from 33% for the 3550 bp LacZ insert to 75% for the 3263 bp UG3.3 insert. These variations in DNA recovery were similar to those obtained in our laboratory.

Yields of Miniprep DNA Isolated

Lower yields of plasmid pUG400 (ie. 85 ng DNA per ml of LB culture) were obtained from E.coli DH5α cells, compared with plasmids pSPLacZ (ie. 0.5 µg DNA), pSP65 (ie. 1.13 µg DNA) and pUG3.3 (ie. 1.65 µg DNA) isolated in the same way. One reason for this is that the more recent pUC-derived plasmids (pSPLacZ, pUG3.3 and pSP65) are a ColE1 variant of the parental plasmid pBR322. Because they lacked the Rom/Rop gene which negatively controls plasmid replication, plasmid copy number was increased approximately 2-fold (Twigg and Sheratt, 1980; Cesaerni *et al.*, 1982) that of pUG400 (26 copies/genome). Lin-Chao *et al.* (1992) demonstrated that a further increase in copy number of the pUC plasmids (ie. 175 copies/genome) is the result of a point mutation within the replication primer RNA II (Minton *et al.*, 1988; Lin-Chao *et al.*, 1992).

Although pUG400 could have been further amplified by the addition of chloramphenicol, an inhibitor of protein synthesis, this was not done in these small scale isolations.

Increasing the Number of Recombinants Formed in a Ligation Reaction

Generally intramolecular ligation events are a major source of 'background' in blunt-end cloning because vector self ligation produces undesired E.coli transformants (Struhl, 1987). In these experiments, the frequencies of obtaining the desired recombinants were maximized by reducing this source of 'backgound'. This was achieved in two ways as described.

Adjusting the DNA Concentration

Because recombinant plasmids are formed in a 2-step ligation reaction (Struhl, 1987), the concentration of DNA-termini plays a significant role in obtaining the desired recombinants (Sambrook *et al.*, 1989).

Dugaiczyk *et al.* (1975) mathematically proposed an optimal concentration of DNA termini for effective ligations. Two factors called *i* and *j* and their ratio (*j:i*) predict the products formed during the ligation reaction, so that the maximal yield of circular recombinants, containing one insert and one vector molecule is obtained when *i* is 2 to 3-fold larger than *j*. A low *j:i* ratio (ie <1) therefore favours intermolecular ligation, while a high *j:i* ratio (ie. >3) favours intramolecular ligation (Draper *et al.*, 1988). Because the vector DNA had been dephosphorylated in these experiments, a *j:i* ratio of 2 was calculated for the insert DNA only. Because the desired recombinants in these ligation reactions contained one vector and one insert molecule, these DNA fragments were both present in equimolar amounts. In practice, however, the exact quantity of DNA added to a ligation reaction is not known precisely. DNA fragments may have been damaged during their preparation (eg. during dephosphorylation, end-filling 5'-sticky overhangs; isolating gel fragments) and when using DNA isolated by the minipreparation method, it is hard to add the exact amount of DNA to a ligation because of the presence of contaminating molecules makes it impossible to accurately quantify the DNA on a spectrophotometer.

In these experiments, only the transformants containing vector sequences were selected using ampicillin resistance. *E. coli* cells that were not transformed with DNA or contained only insert sequences were not able to inactivate the ampicillin and so were not selected for on this media. In addition insert fragment do not contain an origin of replication and so are unable to be maintained by the bacterium (Liew, 1992). Because the insert fragments used in these ligations contained blunt-ended termini and could self-ligate, they may have indirectly contributed to the 'background' level of undesired transformants; because a self-ligated insert is unavailable for ligation to vector DNA. A higher molar concentration of insert DNA in the ligation reaction would have favoured intermolecular ligation events between the vector and insert; this would be particularly true for the small insert fragments'

eg UG400 (Struhl, 1987; Sambrook *et al.*, 1989; Bercovich *et al.*, 1992). Because vector DNA used in these experiments were dephosphorylated, this source of 'background' may not have been such a problem in the ligation reaction.

Dephosphorylation

The 5'-phosphate groups were removed from both termini of the linearized vector DNA in a process called dephosphorylation. This favoured intermolecular ligation between the insert and vector because only DNA fragments containing 5'-terminal phosphates are able to be ligated to the dephosphorylated vector. This promotes intermolecular ligation between insert and vector DNA and reduces the background intramolecular ligation of vector sequences only. The resulting open circular molecule containing 2 single-stranded nicks are repaired after introduction into 'competent' *E. coli* cells and are transformed almost as efficiently as closed circular DNA.

T4 DNA Ligase

Ligation reactions were run below the temperature optima for the enzyme T4 DNA ligase. This is because the rate limiting step in a blunt-end ligation reaction is the association of their end with one another. Bercovich *et al.* (1992) found that optimal conditions for T4 DNA ligase were 100 units ml^{-1} (500 Weiss units' ml^{-1}) and incubated for either 5 hours at 30°C or 15 hours at 15°C. In these experiments, 100 units' ml^{-1} of T4 DNA Ligase were used and incubated for 20 hours at 22°C.

2.11.2 Transformation

The desired recombinants containing one insert and one vector were identified from the 'background' of self-ligated vector using restriction endonuclease cleavage. *E. coli* transformants containing the desired recombinants could not be identified on the LB agar plates. Instead transformants were randomly picked from the plate for miniprep analysis.

In blunt-end ligation the insert can be cloned in either one of two possible orientations within the vector. These two possibilities were distinguished from one another using the appropriate restriction endonuclease(s) that cleaved asymmetrically within the insert DNA.

In these ligations, the transformation efficiency of 'competent' *E.coli* DH5α cells were calculated as the number of colonies obtained per μg of DNA transformed. However accurate transformation efficiencies can only be obtained if: (1) the quantity of DNA transformed is precisely known and; (2) no competition exists between plasmid DNA for the same 'competent' cell. Within a population of 'competent' E.coli cells, only a few are capable of taking up DNA, even if excess DNA is added to the transformation reaction.

The transformation efficiency of E.coli strain DH5α cells used in these ligation reactions was 6.8 x 10^6 colonies' μg^{-1} of pSK Bluescript. This was similar to values reported by other authors (Sambrook *et al.*, 1989). Because there was a theoretical total of 1.25 x 10^8 to 1.75 x 10^8 cells present in a 100 μl aliquot of competent cells, it is calculated that only 3.9% of the available DH5α cells were 'competent' for DNA uptake. These values were similar to those reported by other authors and to that obtained in our laboratory.

In these ligations the transformation efficiency of the covalently closed circular vectors pSP65 and pSPLacZ, isolated using the alkaline lysis miniprep procedure (control #2) ranged from 6.7 x 10^5 colonies μg^{-1} of pSP65 (Table 2.1) to 2.4 x 10^5 and 4.2 x 10^5 colonies μg^{-1} of pSPLacZ (Tables 2.2 and 2.3 respectively). These efficiencies were approximately 10-fold lower than for the pure preparation of pSK Bluescript, suggesting that in these ligations miniprep DNA being impure, may have inhibited transformation.

2.11.3 Overall Ligation and Transformation Efficiencies

Cloning #1: Cloning of *E. coli* LacZ Insert into pSP65 Vector (Table 2.1).

In this ligation, there was a high background of uncleaved vector DNA (Control #3) which was reflected in a high 'background' of undesired transformants containing only vector sequences.

Vector DNA had been gel purified by DEAE-cellulose membrane and then linearized with the appropriate restriction endonuclease. Although this uncleaved DNA could not be visualized at the time using agarose gel electrophoresis, they would have still been highly transformable.

In these experiments, intramolecular ligation was good. At least 45% of the DNA was present in a transformable circular form at the end of the ligation reaction (control #4).

The efficiency of the dephosphorylation reaction was good (control #5); there was a 94.6% inhibition of self-ligation through removing the phosphate groups from the vector DNA.

Two recombinants out of 20 from the experimental contained the LacZ insert within pSP65 which represented a transformation efficiency of 487.8 colonies μg^{-1} of DNA. Both transformants were oriented in the desired 5'-3' position within pSP65. The frequency of obtaining the correct recombinant was 17%.

Cloning #2: Cloning of UG400 Insert into pSPLacZ Vector (Table 2.2).
In this ligation there were no colonies from both the linearized pSPLacZ vector (control #3) and vector religated (control #4). The reasons for this are unknown. Vector DNA had been linearized with the appropriate restriction endonuclease, ensuring that this restriction reaction went to completeness.

Although the vector sequence did not religate they ligated to the insert DNA. Like cloning #1, there was a high background of vector self-ligated in this experiment. Only one recombinant out of the 16 contained a UG400 and this was oriented in the desired 5'-3' orientation within LacZ. The frequency of obtaining the desired recombinant was 6.7%.

Cloning #3: Cloning of UG3.3 Insert into pSPLacZ Vector (Table 2.3).
In this ligation there were no colonies for pSPLacZ religated (control #4). The reasons for this remain unclear because they ligated to the UG3.3 insert.

Only one recombinant out of 19 contained a UG3.3 insert and this was oriented in the desired 5'-3' orientation within pSPLacZ. The frequency of obtaining the desired recombinant was 5%.

2.11.4 Conclusions

In all 3 ligations reactions there was a high background of self ligated vector. Although the reasons for this could not be established in clonings #2 and #3, for #1, the appropriate controls suggested that it was due to the presence of uncleaved vector.

CHAPTER 3

EXPRESSION OF PLASMID DNA IN MOUSE ZYGOTES

3.1 Introduction

Expression of genes in mammalian cells is not limited to eukaryotic genes. In particular, prokaryotic genes under the control of eukaryotic regulatory elements have been extremely useful in the investigation of genes of interest whose product cannot be assayed easily. These 'reporters' of gene activity include the bacterial enzymes chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), β-glucuronidase (GUS); Firefly luciferase; herpes simplex virus thymidine kinase (HSV-tk).

In this chapter, plasmids pUG3.3LacZ and pUG400LacZ were microinjected into the nuclei of mouse zygotes to detect expression from rabbit uteroglobin at the the 2-cell stage of development.

This chapter is divided into 2 parts. In the first, the materials and methods are described while in the second the results obtained are described and discussed.

3.2 Materials

3.2.1 Gonadotrophin Hormones

Pregnant mare serum gonadotrophin (PMSG; 1000 IU) and human chorionic gonadotrophin (hCG; 2500 IU) hormones used for superovulation, were supplied from Sigma as a lyophilized powder. PMSG was resuspended at 500 $IUml^{-1}$ in sterile 0.9% NaCl and was stored in 100 µl aliquots for a maximum of 3 weeks at -20 °C. To administer the hormone, a 100 µl aliquot (500 $IUml^{-1}$) of PMSG was resuspended at 50 $IUml^{-1}$ in sterile 0.9% NaCl. A dose of 0.1 ml (5 IU) was injected intraperitoneally (i.p) into individual mice.

hCG was resuspended at 500 $IUml^{-1}$ in sterile double distilled (dd) H_2O, divided into 100 µl aliquots, lyophilized on a speedvac concentrator (Salvant Instruments Co;

Model RH-40-11, USA) and then stored at -20 °C. To administer the hormone, a 100 µl aliquot (500 $IUml^{-1}$) was resuspended in 1 ml of 0.9% NaCl to give a final concentration of 50 $IUml^{-1}$. A dose of 0.1 ml (5 IU) was injected i.p into individual mice.

3.2.2 Mice

Six week old Swiss Random White (SRW) female mice, purchased from the Animal Breeding Station, Mosgiel or bred from their stock, in our colony, were used in all experiments. Purchased mice were given a few days to adjust to the animal house light/dark cycle before being superovulated. All mice were housed in a day/night controlled room with a 5 am-7 pm light cycle.

3.3 Methods

3.3.1 Equipment

Transfer Pipettes

In these experiments, transfer pipettes controlled by a mouth tube were used for the collection and handling of the mouse zygotes and 2-cell embryos. These were made by 'pulling' the stem of a Pasteur pipette (BDL 4642) in the flame of a Bunsen burner so that its internal diameter (~100 um) was slightly larger than the diameter of the embryos themselves.

Injection Needles

Injection needles were used for microinjecting plasmid DNA into the pronuclei of mouse zygotes. Glass capillary tubing (World Precision Instruments, USA) with an outside diameter of 1 mm, was 'pulled' on a Flaming/Brown Micropipette Puller (Settings: Heat 400, Pull 250, Velocity 100, Time 180 msec; Sutter Instrument Co, Model P-87, San Rafael, CA 94912) to produce an ultra-fine tip suitable for microinjection (Flaming and Brown, 1982).

Holding Needles

Holding needles were used to immobilize the zygote when microinjecting. Accu-fill

90 disposable pipettes (Goldseal Glassware, USA) were 'pulled' on a Flaming/Brown Micropipette Puller (Settings: Heat 400, Pull 100, Velocity 200, Time 190 msec).

Using standard techniques (Hogan *et al.*, 1986), and a microforge heating filament (Technical Products International, Inc. USA), holding needles were then broken cleanly, so that at their tip, their outside diameter was approximately 80 um. This end was then melted to reduce its opening to an internal diameter of about 15 um.

To facilitate the movement of zygotes around the injection chamber, the holding needle was bent to an angle of approximately 15°, 2-3 mm from its end, using the microforge heating filament.

Stereomicroscope

Dissections of oviducts, collection and staining of zygotes and 2-cell embryos were carried out using a stereodissection microscope (Nikon SMZ-2T) with a transilluminator base, using 25-63X magnification.

3.3.2 Plasmid DNA Preparation

DH5α cell containing plasmids p610ZA, pSPLacZ, pUG400LacZ and pUG3.3LacZ were each grown in 500 ml of Terrific Broth medium (Sambrook *et al.*, 1989), supplemented with 60 $\mu g ml^{-1}$ ampicillin. These were shaken at 225 rpm at 37 °C for 17-20 hours until the *E.coli* cells were visually assessed to have entered the stationary phase of their growth curve (Sambrook *et al.*, 1989).

The *E.coli* cells were then harvested in 250 ml polycarbonate bottles in a GSA rotor (DuPont Instruments, USA) at 5 900 g for 15 minutes at 4 °C in a centrifuge (Sorvall^{R} RC-5 Superspeed; DuPont Instruments, USA). The plasmid DNA was isolated by alkaline lysis using a protocol modified by Sambrook *et al.* (1989), derived from the methods reported by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

The closed circular plasmid DNA was purified on a caesium chloride-ethidium bromide equilibrium gradient (Sambrook *et al.*, 1989) by ultra-centrifugation (Beckman L8-70; USA), using 33 ml polyallomer ultra-centrifuge tubes (Nalgene^{R};

USA) and a Ti70.1 rotor. Ultra-centrifugation was for 24 hours at 142 000 g and for a further 36 hours at 106 000 g at 20 °C. Collection of the closed circular plasmid DNA from the ultra-centrifugation tubes and the removal of ethidium bromide using isoamyl alcohol were as described by Sambrook *et al* (1989).

Plasmid DNA was precipitated using 5 volumes of precipitation buffer (0.4 M NaCl; 1 mM EDTA; 10 mM Tris pH 7.2) and 2 volumes of absolute ethanol, and left overnight at -20 °C. This was then centrifuged at 7 600 g for 30 minutes at 4 °C in a SS34 rotor (DuPont Instruments; USA). The DNA pellet was dissolved in 4 ml of precipitation buffer and again precipitated in 2.5 volumes of absolute ethanol for 1 hour at -100 °C. This was then centrifuged in a SS34 rotor at 12 000 g for 30 minutes at 4 °C. The pellet was vacuum dried on a speedvac concentrator and resuspended in 500 µl of TE.

The identities of the isolated plasmids were verified with the appropriate diagnostic restriction endonuclease and the DNA fragments analyzed after separation through an agarose gel (Section 2.3.3). The amount of plasmid DNA was accurately quantified with spectrophotometer (Section 2.3.4).

Plasmids p610ZA, pSPLacZ, pUG400LacZ and pUG3.3LacZ were linearized with the appropriate restriction endonuclease. These were then deproteinized with 1 volume of phenol (pH 8.0):chloroform:isoamyl alcohol (25:24:1), according to the methods of Sambrook *et al.* (1989); repeated until no denatured protein was present at the interface between the organic and aqueous phases. The DNA was recovered by precipitation according to Section 2.3.4. The amount of plasmid DNA was accurately quantified on a spectrophotometer (Section 2.3.4).

3.3.3 Media

Recovery and manipulation of zygotes and 2-cell embryos were carried out in Hepes-buffered Tyrodes (HT6) medium (pH 7.4; 285-287 mosmol), containing 4.95 $mgml^{-1}$ bovine serum albumin (BSA; Gibco Fraction V). BSA was added to the medium on the day of microinjection (Hogan *et al.*, 1986; originally reported by Quinn *et al.*, 1982), to both reduce the stickiness of the embryos and to adsorb (or supply) trace contaminants (or requirements) in the medium (Hogan *et al.*, 1986). All media were

made under sterile conditions using sterilized double-distilled (dd) H_2O (demineralized on a combined activated-charcoal/mixed bed ion exchange system; Sybron/Barnstead NanopureII cartridge system) using chemicals of tissue culture grade. Solutions were filtered using positive pressure through a sterile 0.22 um filter (Cameo IIS, MSI, Westborough, MA, USA), discarding the first few millilitres and then aliquoted into sterile disposable polystyrene 15 ml (Falcon^R 2095) tubes and stored at 4 °C for up to 1 week.

3.3.4 Embryo Culture

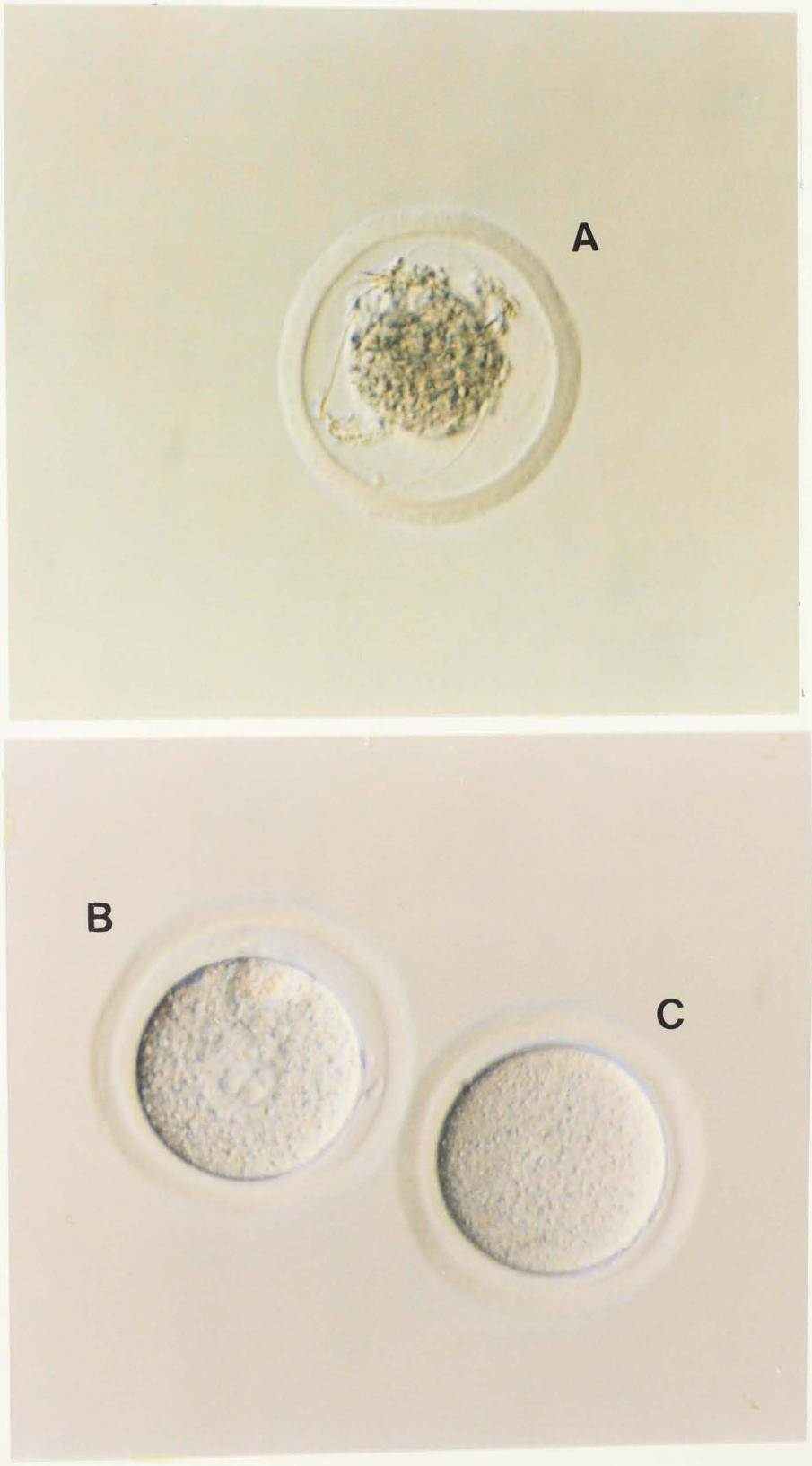
Before and after microinjection, zygotes were kept in a closed incubator (Sanyo) in 5.1% $C0_2$ in air at 37 °C. To prevent evaporation of the media and contamination from surrounding air, the embryos were cultured in 50 µl drops of HT6 media in sterile culture dishes (Falcon^R 3002), overlaid with pre-equilibriated Dow Corning^R 200 silicon oil (Viscosity Factor of 50, Specialized Products Co, USA) as described by Hogan *et al.* (1986) and originally reported by Brinster (1963). These tissue culture dishes were set up a short time in advance at 37 °C and 5.1% CO_2 before adding the embryos, to allow for pre-equilibrium of temperature and CO_2.

3.3.5 Embryo Collection

Zygotes were obtained from 6 week old random bred Swiss White (SRW) female mice. These mice were superovulated by an ip of 0.1 ml (5 IU) PMSG between 10 am-2 pm, followed by 0.1 ml (5 IU) i.p of hCG 42-46 hours later (Hogan *et al.*, 1986). Eight hours after the hCG injection, these mice were individually paired overnight with fertile males of the same strain, to obtain embryos.

Zygotes (fertilized ova) were flushed from excised oviduct(s), from females which had copulation plugs 24-26 hours post-hCG (Hogan *et al.*, 1986). Zygotes were distinguished from unfertilized ova by the presence of 2 polar bodies and the presence of a male and female pronucleus (Hogan *et al.*, 1986; Figure 3.1). Attached cumulus oophorus cells were dispersed with hyaluronidase (1.6 $mgml^{-1}$ of HT6 media; Boehringer Mannheim), for 1-2 minutes until the cumulus cells were released. These zygotes were then washed in 2 x 2 ml changes in HT6 media.

Figure 3.1 (A) Zygote lysed during microinjection; (B) Mouse zygote; (C) Unfertilized ovum.



3.3.6 Microinjection

DNA Preparation for Microinjection

The plasmid DNA to be microinjected into the zygotes was microcentrifuged at 13 000 g for 10 minutes, to remove any particulates that could clog the injection needle. One hundred nanograms of the plasmid DNA to be microinjected (50 $ng\mu l^{-1}$ p610ZA; 85 $ng\mu l^{-1}$ pUG3.3LacZ; 100 $ng\mu l^{-1}$ pUG400LacZ; 120 $ng\mu l^{-1}$ pSPLacZ) was removed and placed in 50 µl of MTE [10 mM Tris.Cl (pH 8); 0.25 mM EDTA; filter sterilized] and micro-centrifuged for a further 30 minutes at 13 000 g. The top 25 µl of this solution was used for microinjection and contained the plasmid DNA at a concentration of 2 μgml^{-1}.

Injection Chamber

The zygotes to be microinjected were transferred into a 25 µl flattened microdrop of HT6 media, in a sterile inverted lid of a (Falcon^R 1006) Petri dish and overlaid with pre-equilibrated Dow Corning^R 200 silicon oil.

Microinjection of DNA in Mouse Zygotes

Zygotes were microinjected at 400X magnification using a Hoffman Modulation Contrast 40X objective and 10X eyepiece on an inverted microscope (Nikon Diaphot). Micromanipulators (Narashige, USA), were mounted on each side of the microscope; one controls the holding needle and the other the injection needle. The holding needle was controlled using a compressed oil system and the injection needle by a compressed air system (Hogan *et al.*, 1986). The injection needle was filled with the plasmid DNA (2 μgml^{-1}) in MTE [10 mM Tris.Cl (pH 8.0); 0.25 mM EDTA] by capillary action. The zygotes were set up in the injection chamber as described and the holding and injection needles were set up in the injection chamber using the methods of Hogan *et al.* (1986).

Using the micromanipulators and under 400X magnification, the tip of the injection needle was opened by brushing it gently against the holding needle until a hole of sufficient size was made (Hogan *et al.* 1986), so that DNA solution flowed out freely under pressure.

Each zygote was held in place by suction applied through the holding needle. Plasmid DNA was forced out of the tip of the injection needle and microinjected into one of the pronuclei (Hogan *et al.* 1986); the rate of flow being limited by the size of the hole. Successful injection was shown by momentary swelling of the pronuclei in response to the pressure applied.

After microinjection, zygotes were transferred in a 1 x 2 ml of HT6 media to remove any silicon oil traces and cultured for 20-24 hours to the late 2-cell stage (Section 3.3.4). At the end of the culture period, embryos were scored for their developmental stage (ie. degenerative embryo; 1-cell; 2-cell etc), before measuring β-galactosidase activity in individual embryos.

3.3.7 Histochemical Assay for β-galactosidase Activity in 2-Cell Embryos

Stock solutions of 99 mM of potassium ferrocyanide (wrapped in foil) and 102 mM potassium ferricyanide were prepared in dd H_2O and stored at room temperature in Falcon^R (2095) tubes. Stock solutions of one times phosphate buffered saline (1X PBS; 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4 pH 7.3) and 1 M $MgCl_2$ were stored at room temperature. 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal; Sigma) was dissolved in dimethyl formamide (Sigma) at 20 $mgml^{-1}$ and stored in its glass container wrapped in foil at -20 °C. Fixative solution (1% formaldehyde, 0.2% glutaraldehyde, 0.02% nonidet P-40, 1 mM $MgCl_2$ in 1X PBS pH 7.3) was stored at 4 °C for a maximum of 2 weeks.

Individual embryos were histochemically assayed for cytoplasmic detection of β-galactosidase, 20-24 hours post-microinjection (44-50 hours post-hCG). The following histochemical staining procedure was supplied by Kennedy, (1989). Embryos were transferred sequentially into:

1. 50 µl of HT6 media and then into 50 µl of 1X PBS (pH 7.3).
2. 1 x 2 ml of fixative solution for 5 minutes at 4 °C.
3. 6 x 50 µl drops of 1X PBS (pH 7.4) to remove any traces of fixative solution.
4. 50 µl of fresh X-gal staining solution (4.7 mM potassium ferrocyanide, 4.85 mM potassium ferricyanide, 0.95 mM $MgCl_2$, 0.5 mg of 20

mgml^{-1} X-gal in 0.85X PBS), in a sterile tissue culture dish (Falcon 3002), overlayed with Dow Corning^{R} silicon oil to prevent dessication. These were kept at 37 °C for 24 hours in a closed incubator (Sanyo) in 5.1% $C0_2$ in air (Section 3.3.4).

Control and injected embryos were treated equivalently.

3.3.8 Statistical Analysis

To control for variations in microinjection efficiencies and the number of zygotes superovulated at any one time, each linearized plasmid, pSPLacZ, p610ZA, pUG400LacZ and pUG3.3LacZ were randomly assigned to microinjection on different days, one plasmid per day, with 4 repetitions, using randomization tables and methods of Snedecor and Cochran (1982).

Control mouse zygotes not microinjected were used on each microinjection day to check that positive β-galactosidase staining could not be attributed to endogenous mammalian β-galactosidase activity (Cojda, 1970; Nanba and Suzulci, 1990; Cecilia *et al.*, 1991). These embryos were treated similar to the microinjected embryos except that they were not microinjected with plasmid DNA.

3.4 Results

3.4.1 Preparation of Plasmid DNA for Microinjection

pUG3.3LacZ

Purification of pUG3.3LacZ on a caesium chloride-ethidium bromide density gradient yielded 1205 µg of plasmid per 500 ml of Terrific Broth (ie. 2.41 µg of plasmid ml^{-1} of Terrific Broth), with an A_{260}/A_{280} ratio of 1.4. To verify its identity, an aliquot of this DNA was cleaved with 10 units of Stu I and 10 units of Eco RV as a double restriction digest. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave the expected DNA fragment lengths of 345, 3181 and 6374 bp as shown on the plasmid map in Figure 2.11.

Plasmid pUG3.3LacZ was linearized with Spe I (Figure 2.11), and the linearized plasmid was microinjected into the pronuclei of mouse zygotes.

pSPLacZ

The purification of pSPLacZ on a caesium chloride-ethidium bromide density gradient yielded 498.1 µg of plasmid per 500 ml of Terrific Broth (ie. 0.996 µg of plasmid ml^{-1} of Terrific Broth), with an A_{260}/A_{280} ratio of 1.44. To verify its identity, an aliquot of this DNA was cleaved with 10 units of Eco RI. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave the expected DNA fragment lengths of 3084 and 3505 bp (Figure 2.3).

Plasmid pSPLacZ was linearized with Xba I (Figure 2.3), and the linearized plasmid was microinjected into the pronuclei of mouse zygotes.

pUG400LacZ

The purification of pUG400LacZ on a caesium chloride-ethidium bromide density gradient yielded 506.1 µg of plasmid per 500 ml of Terrific Broth (ie. 1.01 µg of plasmid ml^{-1} of Terrific Broth), with an A_{260}/A_{280} ratio of 1.47. To verify its identity, an aliquot of this DNA was cleaved with 8 units of Bgl I. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave the expected DNA fragment lengths of 2814 bp, 530 bp, 2121 bp and 1599 bp as shown on the plasmid map in Figure 2.7.

Plasmid pUG400LacZ was linearized with Pst I (Figure 2.7) and the linearized plasmid was microinjected into the pronuclei of mouse zygotes.

p610ZA

The purification of p610ZA on a caesium chloride-ethidium bromide density gradient yielded a total of 1090 µg of plasmid per 500 ml of Terrific Broth (ie. 2.18 µg of plasmid ml^{-1} of Terrific Broth), with an A_{260}/A_{280} ratio of 1.5. To verify its identity, an aliquot of this DNA was cleaved with Pst I and Kpn I. Electrophoresis of this restricted DNA on a 0.8% agarose gel gave the expected DNA fragment lengths of 3850 bp and 2680 bp (Figure 2.1).

Plasmid p610ZA was linearized with Kpn I (Figure 2.1) and the linearized plasmid was microinjected into the pronuclei of mouse zygotes.

3.4.2 Experimental Methodology

Each of the linearized plasmids, pUG3.3LacZ, pUG400LacZ, pSPLacZ and p610ZA were randomly assigned to microinjection days (Section 3.3.8). A total of 120-150 mouse zygotes were microinjected with each plasmid.

Expression of the LacZ reporter gene from the constructs pUG400LacZ, pSPLacZ, pUG3.3LacZ and pUG400LacZ were detected using a sensitive enzyme in situ detection procedure for *E. coli* β-galactosidase. The chromogenic substrate, X-gal was enzymatically cleaved by β-galactosidase to release an insoluble blue dye. This allowed a direct visual confirmation of promoter activity in the intact embryo detected by the presence of a dark blue precipitate in either one or both blastores. The expression efficiencies of the different constructs were quantified by:

(1) by calculating total fractions of β-galactosidase-positive embryos.

(2) classifying embryos into four arbitrary classes of staining intensity representing different levels of β-galactosidase activity (Figure 3.2).

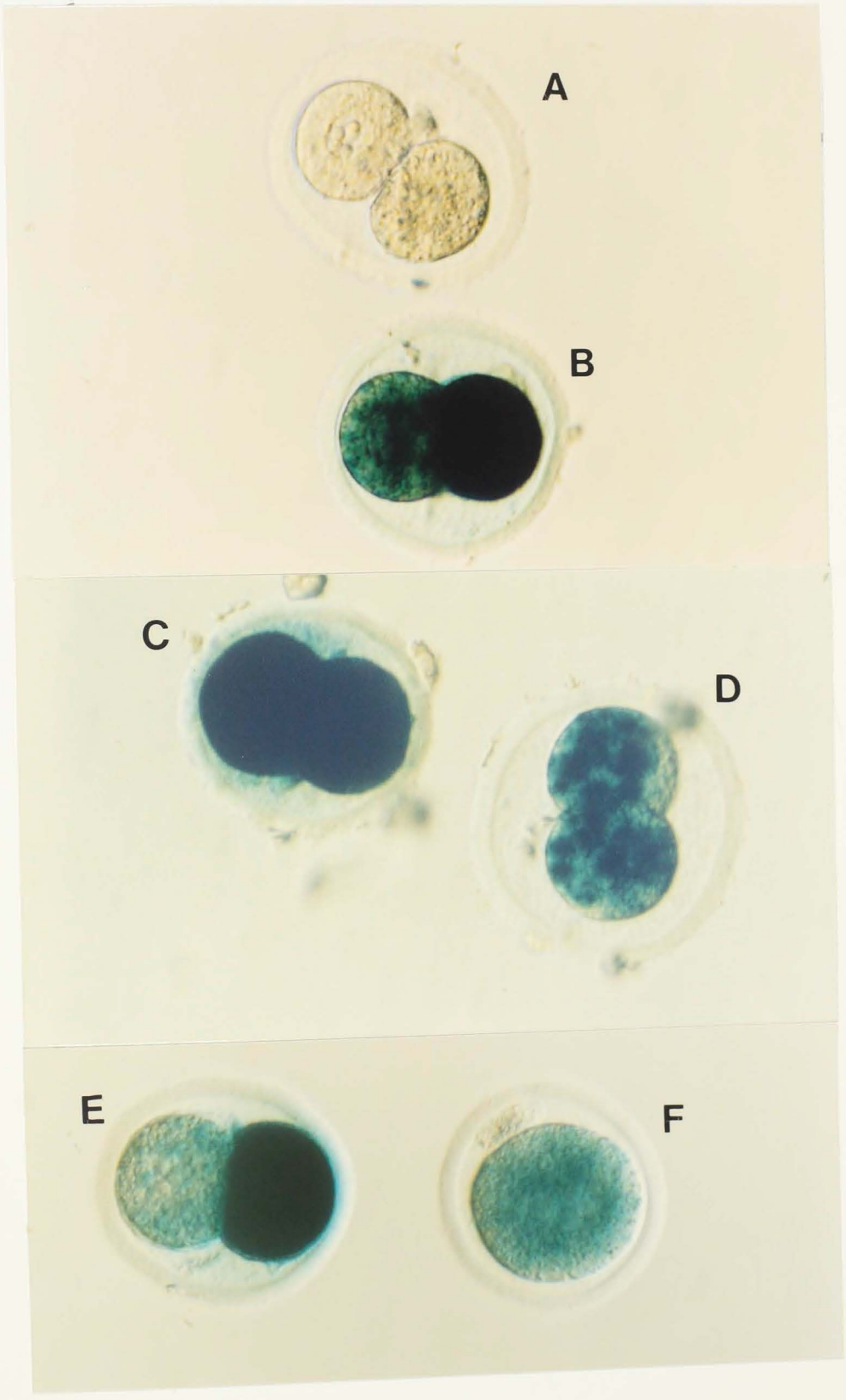
3.4.3 Preliminary Results using Plasmid p610ZA

An *E.coli* LacZ vector containing 300 bp of 5'-flanking mouse heat shock promoter (Hsp70) fused to codon 8 of *E.coli* LacZ was microinjected into one of the pronuclei of mouse zygotes.

Superovulation Data

Of the 13 female mice used as egg donors, 11 (85%) had mated with fertile males of the same strain. From these mice, a total of 180 ova were collected of which 131 (73%) were fertilized. This represented an average of 11.9 zygotes per plugged donor. From the 131 zygotes collected, 84 were microinjected and 33 were used as non-microinjected controls. Fifty-nine (70%) of the zygotes survived microinjection and from these 37 (63%) continued development to the 2-cell; the remainder arrested in development at the 1-cell. Of the cultured non-microinjected zygotes, 31 (94%) continued development to the 2-cell (Table 3.1).

Figure 3.2 Expression of p610ZA (Hsp70-LacZ) in mouse embryos
(A) Control (not injected) embryo; (B-E) 2-cell embryos showing different levels of staining; (F) 1-cell embryo.



Microinjection Data

These preliminary experiments verified that this Hsp70-LacZ reporter construct was efficiently expressed in both 2-cell embryos and those arrested in development at the 1-cell stage (Table 3.1). Of the 25 2-cell embryos, 24 (96%) expressed β-galactosidase, as did 88% of those arrested in development at the 1-cell stage. LacZ β-galactosidase activity was not observed in control embryos that were not microinjected. β-galactosidase positive embryos displayed various degrees of staining, ranging from scattered patches of blue dye in clear cytoplasm, to a homogeneously distributed, dark indigo stain (Figure 3.2).

Table 3.1 Preliminary Superovulation and Microinjection Data Using Plasmid p610ZA.

No. Donors	Plugged Donors	(%)	No. Ova Collect.	No. Fertil.	(%)	No. Inject.	No. Died	(%)	1-Cell Positive	(%)	2-Cell Positive	(%)
4	4	(100)	57	54	(95)	26	15	(58)	4/4	(100)	6/6	(100)
3	3	(100)	38	31	(82)	23	2	(8.7)	10/10	(100)	8/8	(100)
3	3	(100)	48	26	(54)	20	4	(20)	0/1	(00)	3/3	(100)
3	1	(33)	37	20	(54)	15	4	(27)	1/2	(50)	7/8	(88)
Totals 13	11	(85)	180	131	(73)	84	25	(30)	15/17	(88)	24/25	(96)

Table 3.2 Superovulation Data.

	No. Donors	Plugged Donors	(%)	No. Eggs Collected	No. Fertil.	(%)	Zygotes/ Pl. Donor
p610ZA	15	11	(73)	222	108	(81)	16.3
pSPLacZ	13	10	(77)	246	162	(66)	16.2
pUG400LacZ	13	9	(69)	236	178	(75)	19.6
pUG3.3LacZ	15	12	(80)	210	150	(71)	12.5
Overall	56	42	(75)	914	73		15.9

3.4.4 Expression of Plasmids pUG400LacZ, pUG3.3LacZ, pSPLacZ and p610ZA

The expression of pUG3.3LacZ and pUG400LacZ in 2-cell mouse embryos was compared with p610ZA and the 'promoterless' LacZ reporter gene, pSPLacZ.

Superovulation Data

Of the 56 female mice used as egg donors, 42 (75%) had mated with fertile males of the same strain. From these 914 ova were collected, of which 670 (73%) were fertilized. This represented an average of 15.9 zygotes per plugged donor (Table 3.2).

Microinjection Data

From the 670 zygotes collected, 557 were microinjected and 77 were assigned to the control non-microinjected group. A total of 445 (80%) of the zygotes survived microinjection and from these, 81% continued development to the 2-cell stage, the remainder arrested in development at the 1-cell stage. This compared well with the control non-microinjected zygotes of which 72 (94%) continued development to 2-cell (Table 3.3)

Expression of β-galactosidase

1. p610ZA

Of the 15 female mice used as egg donors, 11 (73%) had mated with fertile males. From these mice, a total of 222 ova were collected of which 180 (81%) were fertilized, representing an average of 16.3 zygotes per plugged donor (Table 3.3).

One hundred and ten (75%) zygotes survived microinjection and from these 82 (75%) continued development to 2-cell. Of the cultured non-microinjected zygotes, 20 (87%) continued development to 2-cell.

Of the 1-cell embryos arrested in development, 22 out of 27 (82%) expressed β-galactosidase, whereas 86% of the 2-cell embryos expressed β-galactosidase (Figure 3.2; Table 3.3). LacZ-β-galactosidase was not observed in control embryos that were not microinjected.

2. pUG3.3LacZ

Of the 15 female mice used as egg donors, 12 (80%) had mated. From these mice, a total of 210 ova were collected of which 150 (71%) were fertilized, representing an average of 12.5 zygotes per plugged donor (Table 3.2). From these 150 zygotes collected, 119 were microinjected and 16 were used as non-microinjected controls.

Ninety zygotes (76%) survived microinjection and from these 77 (86%) continued development to 2-cell. Of the cultured non-microinjected zygotes, 15 (94%) continued development to 2-cell.

Plasmid pUG3.3LacZ did not express in 2-cell embryos or those arrested in development (Table 3.3; Figure 3.3).

3. pUG400LacZ

Of the 13 female mice used as egg donors, 9 (69%) had mated. From these mice, a total of 236 ova were collected of which 178 (75%) were fertilized, representing an average of 19.6 zygotes per plugged donor (Table 3.2). From the 178 zygotes collected, 148 were microinjected and 20 were used as non-microinjected controls.

One hundred and thirty three zygotes (90%) survived microinjection and from these, 119 continued development to 2-cell. Of the cultured non-microinjected zygotes, 20 (100%) continued development to 2-cell.

Plasmid pUG400LacZ did not express in any of the 2-cell embryos or those arrested in development (Table 3.3; Figure 3.3).

4. pSPLacZ

Of the 13 female mice used as egg donors, 10 (77%) had mated. From these mice, a total of 246 ova were collected of which 162 (66%) were fertilized, representing an average of 16.2 zygotes per plugged donor (Table 3.2). From the 162 zygotes collected, 144 were microinjected and 18 were used as non-microinjected control.

One hundred and twelve zygotes (78%) survived microinjection and from these, 83 (74%) continued development to 2-cell. Of the cultured non-microinjected zygotes,

17 (94%) continued development to 2-cell.

None of the 2-cell embryos or zygotes that lysed during microinjection expressed β-galactosidase. One of the 29 (03%) 1-cell embryos that were arrested in development expressed β-galactosidase (Table 3.3; Figure 3.4).

Figure 3.3 Non-expression of pUG400LacZ and pUG3.3LacZ in mouse embryos.

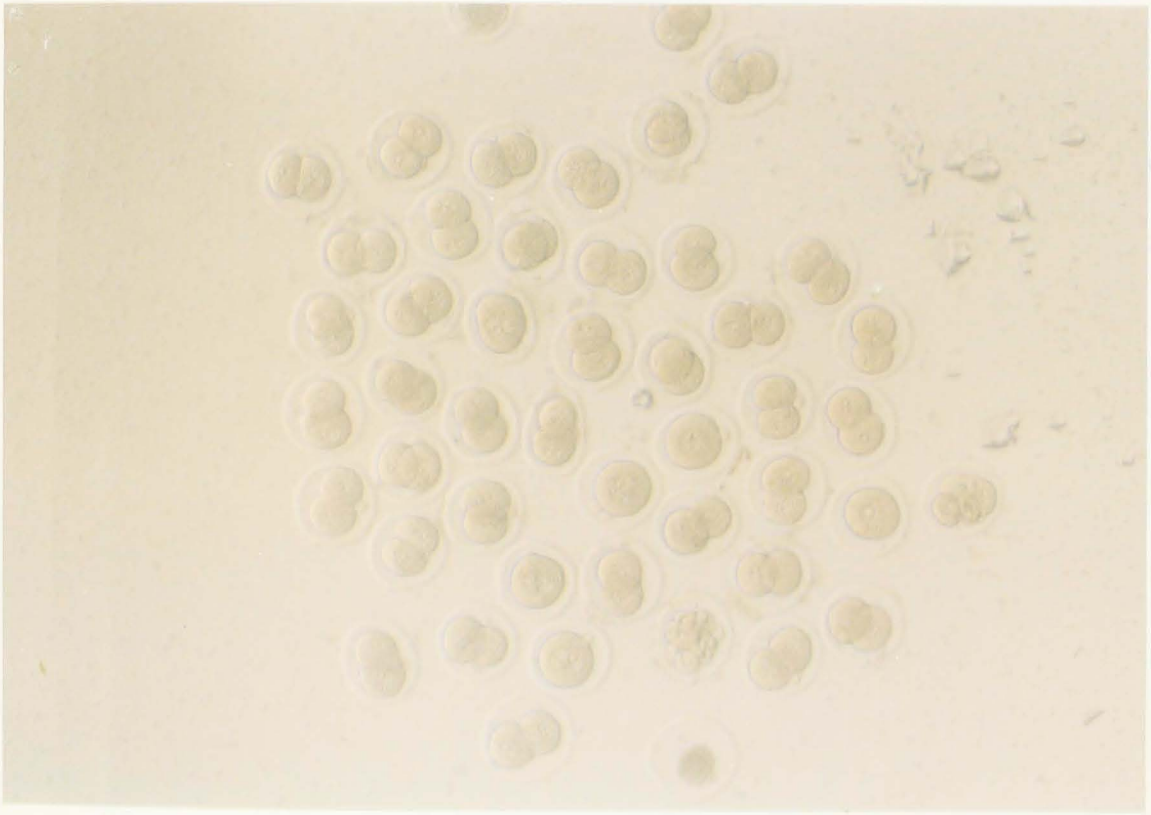
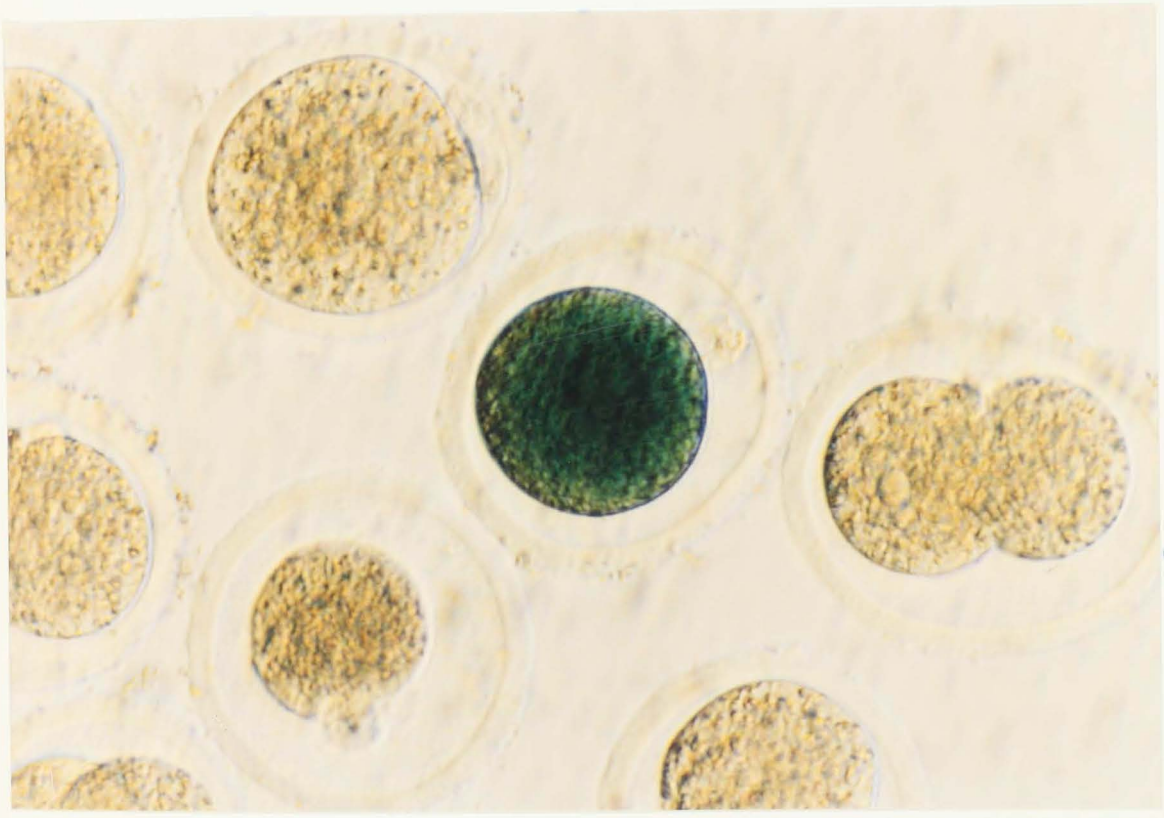


Figure 3.4 Expression of pSPLacZ in 1-cell mouse embryo.



3.5 Discussion

3.5.1 Constructs

Constructs pUG400LacZ, pUG3.3LacZ were microinjected into the pronuclei of mouse zygotes and their expression at the 2-cell stage was compared to pSPLacZ, a promoterless LacZ cloning vector and p610ZA which was shown in preliminary studies to express efficiently at this stage of development.

Although p610ZA (Hsp70-LacZ) expressed efficiently in both 2-cell embryos and those that arrested in development at the 1-cell stage, no expression was detected from constructs pUG3.3LacZ and pUG400LacZ. Surprisingly, pSPLacZ was unambiguously expressed in a 1-cell embryo that had arrested in development. One possible reason for this is that this LacZ reporter gene containing a 5' Kozak translation initiation sequence integrated into the genome at a position where it was transcribed under the 5' regulatory elements of a promoter that was active at this stage of development.

Expression of DNA at this stage of development is mainly episomal (Kothary *et al.*, 1989). It is thought that at least up to the 8-cell stage, episomal copies of the injected DNA still exists. Because this DNA is not integrated into the genome, microinjected DNA sequences are free from 'position effects' that act on integrated genes. Plasmid p610ZA showed a mosaic pattern of expression where one blastomere would express β-galactosidase at a different level than the other. Presumably this reflects the episomal nature of the DNA. During cleavage there would be unequal segregation of the plasmid DNA between the 2 blastomeres.

3.5.2 Factors Affecting Gene Transfer

Microinjection

In this study DNA was microinjected directly into the pronuclei of the mouse zygotes. Factors affecting the microinjection proceedure are discussed below.

DNA Concentration

In these experiments a DNA concentration of 2 μgml^{-1} was microinjected into the

pronuclei, which corresponded to approximately 200-400 molecules for a 5 kb DNA fragment. Brinster *et al.* (1985) found that DNA concentration greater than 10 μgml^{-1} significantly decreased mouse zygote survival but 2-4 μgml^{-1} resulted in an optimal number of transgenic mice.

DNA Purity

DNA samples used for microinjection were purified on a cesium chloride density gradient and microcentrifuged on the day of microinjection to remove contaminants that might harm the egg or decrease embryo survival.

Superovulation

Because it was not important for the genes to be introduced into a defined genetic background, random-bred Swiss (SRW) mice were used in this study because they were a cost-effective strain both in terms of being commercially available in NZ and an increased embryo yield in response to superovulation than the in-bred strains (Brinster *et al.*, 1985). These mice were superovulated prior to mating to both increase the number of ova ovulated and to synchronize the estrous cycle which is normally 4-5 days in mice (Hogan *et al.*, 1986).

The timing of PMSG and hCG administered to each other and to the light cycle of the mouse room affects both the developmental uniformity and the number of eggs recovered from superovulated female mice (Hogan *et al.*, 1986). In this study the mouse house was kept strictly to a 5am to 7pm light cycle to give 14 hours of light and 10 hours of dark. In addition there was a 42-48 hour interval between PMS and hCG injections and this was found to be optimal in terms of egg yield (Hogan *et al.*, 1986).

The best age for superovulation in these mice was found to be at 6 weeks (Damakl, 1991) during the pre-pubescent stage of development. At this stage a wave of follicular maturation has taken place so that the number of follicles capble of responding to FSH at this time is at a maximum.

In this study mice not containing a copulation plug were examined for the presence of fertilized zygotes to see if they had ovulated. This is because the vaginal plug

which usually dissolves at 12-14 hours (Hogan *et al.*, 1986) can sometimes not readily be seen. In addition ovaries were inspected to check that ovulation had occured. Bloody fluid within the ovarian capsule and/or small clots upon the ovaries are often readily visible with the unaided ey and confirm recent ovulation (Rafferty, 1970).

3.5.3 Media

HT6 media was used in this study for the collection and culture of the embryos to the 2-cell stage. It is very similar to Whittens media (Whitten, 1971) except that HEPES buffer was added in the place of some of the bicarbonate in order to help maintain the correct pH of 7.4 when the embryos wer handled for prolonged periods outside of the incubator. In addition, the pH of the medium was maintained between pH 7.2 and 7.4 in culture by incubation at 37 °C in an atmosphere of 5% CO_2 in air.

Because these embryos came from mice that were randomly bred, the majority of the embryos in this study blocked at the 2-cell stage of development.

3.5.4 Fate of DNA in the Nucleus

Chen *et al.* (1986) found that much of the DNA microinjected into the nucleus is still present in its injected form after the zygotes had been cultured for 24 hours. There was a slight conversion from relaxed circular to supercoiled forms of the injected plasmid.

DNA in these experiments were directly microinjected into the pronucleus. Not only is this site where transcription takes place of the inserted gene but is stable for at least 3 days, while DNA injected into the cytoplasm was rapidly degraded (Wirak *et al.*, 1985; Martinez-Salas *et al.*, 1988).

When more than one copy of the DNA integrates, they usually all integrate at the same chromosomal site in a tandem, head-to-tail array, although other arrangements have been documented.

3.5.5 Expression of B-galactosidase

Because expression of DNA microinjected in the zygote can be observed at the 2-cell stage in mice, all the manipulated embryos were subsequently monitored for gene activity at this stage. The expression of the 3 promoters were studied in 2-cell mouse embryos using the expression of *E. coli* LacZ as a reporter of gene function.

E. coli LacZ has been widely used as a reporter of gene activity in a variety of prokaryotic and eukaryotic systems, rivalled only by the *E. coli* Gus gene in plants (Teeri *et al.*, 1989). In these studies the value of the LacZ in comparison to other reporter genes was its ability to visualize gene activity at the cellular level eg. in drosophila embryos (Lis *et al.*, 1983), murine embryos (Bonnerot *et al.*, 1987; Allen *et al.*, 1988; Kothary *et al.*, 1988; Sanes *et al.*, 1986) and rat nervous system (Price *et al.*, 1988).

In these experiments embryos were histochemically assayed for β-galactosidase using X-gal as the chromogenic indicator which is enzymatically cleaved by β-galactosidase to produce an insoluble blue precipitate.

The sensitivity of this staining procedure was increased in these experiments by the incorporation of a fixation step prior to staining (MacGregor *et al.*, 1987). The major components of the stain were:

1. 0.95 mM $MgCl_2$; a cofactor for β-galactosidase activity.

2. Phosphate buffered saline (pH 7.3); the optimal pH for *E. coli* β-galactosidase. By controlling the pH of the stain, no endogenous β-galactosidase activity could be detected.

3. Potassium ferrocyanide (4.7 mM) and potassium ferricyanide (4.85 mM) which acts as an oxidation catalyst to convert the soluble cleavage product of X-gal to an insoluble, exceptionally stable indigo form (Bondi *et al.*, 1982), enhancing localization of the blue precipitate and intensifying its colour (reviewed by MacGregor *et al.*, 1991).

Table 3.3 Expression of β-galactosidase.

Vector	Promoter	No. Injected	No. Died	1-Cell Positive	(%)	2-Cell Positive	(%)
p610ZA	HSP70 (-86/+221)	146	36	22/27	(82)	68/79	(86)
pSPLacZ	None	144	32	1/29	(03)	0/81	(00)
pUG400LacZ	UG400 (-385/+9)	148	15	0/14	(00)	0/119	(00)
pUG3.3LacZ	UG3.3 (-3254/+9)	119	29	0/13	(00)	0/77	(00)
Overall		557	112				

CHAPTER 4
GENERAL DISCUSSION AND CONCLUSIONS

In this study both pUG3.3LacZ and pUG400LacZ did not express at the protein level in either 2-cell embryos or 1-cell embryos arrested in development. There are a number of reasons why this might be the case.

1. At this stage of development the appropriate transcription factors may not have been present. A few studies have suggested that at this stage of development specificity in promoter usage is already present eg. the promoter of the IL-2 gene whose expression is normally restricted to T-cells and the acetylcholine reductase α-subunit (AChR-α) normally restricted to the muscle cells, did not express in 2-cell embryos (Bonnerot et al 1991).

2. Presence of negative transcription factors which may suppress transcription from the uteroglobin promoter.

3. Linerarized constructs containing plasmid DNA were microinjected into the mouse zygotes. Although it has been shown that the presence of plasmid sequences in the transgene may inhibit expression in transgenic mice this has not normally been the case for transient expression studies. For example Stevens et al 1989 found that plasmid sequences did not prevent expression from the metallothionein promoter. Also Bonnerot et al 1991 demonstrated that expression from the B-actin promoter, HPRT promoter and SV40 promoter occured at the same level as those promoters deleted of vector sequences.

4. Plasmid pSP65 may have contained poison sequences which may have prevented expression of the uteroglobin promoter.

5. In these experiments, embryos were assayed for at the translation level. No attempt was made to assay for mRNA levels.

Conclusions

Plasmid p610ZA (Hsp70-LacZ) was efficiently expressed at the 2-cell stage of development. However no expression of pUG400LacZ or pUG3.3LacZ was detected at the translation level. It is likely that tissue specificity in promoter usage occurs at the 2-cell stage of development. Because of the lack of appropriate culture tissue lines to express uteroglobin, these constructs were unable to be checked to see if they were capable of being expressed.

REFERENCES

AATWATER, J.A.; WISDOM, R.; VERMA, I.M. (1990). Reguated mRNA stability. *Annual Review of Genetics* 24 : 519-541.

AUNUKKER, G., SEITZ, J., HEYNS, W., KIRCHER, C. 1985). Ultrastructural localization of uteroglobin immunoreactivity in rabbit lung endometrium and rat ventral prostrate. *Histochemie* 83 : 413-417.

BANERJI, J.; RUSCONI, S.; SCHAFFNER, W. (1981). Expression of a B-Globin gene is enhanced by remote SV40 DNA sequences. *Cell* 27 : 299-308.

BEATO, M., ARNEMANN, J., MENNE, C., MULLER, H., SUSKE, G., WENZ, M. (1983). *Regulation of Gene Expression by Hormones*. Edited by K.W McKerns. New York: Plenum Press.

BEIER, H.M. (1968). Uteroglobin: a hormone sensitive endometrial protein involved in blastocyst development. *Biochemica Biophysica Acta*. 160 : 289-295.

BENSAUDE, O.; BABINET, C,; MORANGE, M.; JACOB, F. (1983). Heat shock proteins, first major products of zygotic gene activity in mouse embryo. *Nature* 305 : 331-332.

BERCOVICH, J.A.; GRINSTEIN, S.; ZORZOPULOS, J. (1992). Effect of DNA concentration on recombinant plasmid recovery after blunt-end ligation. *BioTechniques* 12(2) : 190-193.

BERK, A.J. (1989). Regulation of eukaryotic transcription factors by post-translational modification. *Biochemica et Biophysica Acta* 1009 : 103-109.

BIRNBOIM, H.C.; DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA nucleic acids. *Research* 7 : 1513-1523.

BLOCH, K.D.; BARTOS, B. (1992). Restriction endonucleases, 3.1.1-3.1.20. In: *Current Protocols in Molecular Biology*. Edited by F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Ssiedman, J.A. Smith and K. Struh. Wiley Interscience, New York.

BOEHRINGER MANNHEIM (1989). Personal communication.

BODNER, M.; CASTRILLO, J.-L.; THEILL, L.E.; DEERINCK, T.; ELLISMAN, M.; KARIN, M. (1988). The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* 55 : 505-518.

BOLTON, V.N.; OADES, P.J.; JOHNSON, M.H. (1984). The relationship between cleavage, DNA Replication, and gene expression in the mouse 2-cell embryo. *Journal of Embryology Experimental Morphology* 79 : 139-163.

BRINSTER, R.L. (1963). A method for in vitro cultivation of mouse ova fro two-cell to blastocyst. *Experimental Cell Research* 32 : 205-208.

BROWN, T.A.; CHAPMAN & HALL (eds). Gene cloning: an introduction second edition, 1990

CASADABAN, M.J.; COHEN, S.N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *Journal of Melecular Biology* 138 : 179-207.

CASADABAN, M.J.; MARTINEZ-ARIAS, A.; SHAPIRA, S.K.; CHOU, J. (1983). β-galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and Yeast. *Methods in Enzymology* 100 : 293-308.

CASCIO, S.M.; WASSARMAN, P.M. (1982). Program of early development in the mammal: post-transcriptional control of a class of proteins synthesized by mouse oocytes and early embryos. *Developmental Biology* 89 : 397-408.

CATO, A.C.B.; GEISS, S.; WENZ, M.; WESTPHAL, H.M.; BEATO, M. (1984). The nucleotide sequences recognized by the glucocorticoid receptor in the rabbit uteroglobin gene are located far upstream from the initiation of transcription. *The EMBO Journal* 3 : 2771-2778

CLEGG, K.B.; PIKO, L. (1983). Poly(A) Length, cytoplasmic adenylation and synthesis of poly(A)+ RNA in early mouse embryos. *Developmental Biology* 95 : 331-341.

COHEM, S.N.; CHANG, A.C.Y.; HSU, L. (1972). Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proceedings of the National Academy of Sciences of the USA* 69 : 2110-2114.

COLONNA, R.; CECCONI, S.; TATONE, C.; MANGIA, F.; BUCCIONE, R. (1989). Somatic cell-oocyte interactions in mouse oogenesis: stage-specific regulation of mouse oocyte protein phosphorylation by granulosa cells. *Developmental Biology* 133 : 305-308.

CORDEN, J.; WASYLYK, B.; BUCHWALDER, A.; SASSONE-CORSI, P.; KEDINGER, C.; CHAMBON, P. (1980). Expression of cloned genes in new environment. *Science* 209 : 1406-1414.

COURTOIS, G.; MORGAN, J.G.; CAMPBELL, L.A.; FOUREL, G.; CRABTREE, G.R. (1987). Interaction of a liver-specific nuclear factor with the fibrinogen and a1-antitrypsin promoters. *Science* 238 : 688-692.

COWAN, B. D.; NORTH, D. H.; WHITWORTH, N. S.; FUJITA, R.; SCHUMACHER, E. K.; MUKHERJEE, A. B. (1986). Identification of a uteroglobin-like antigen in human uterine-like washings. *Fertility & Sterility* 45 : 820-823

DAMAK, S. (1991). Personal communication.

DANIELS, D.R.; DE WET, J.R.; BLATTNER, F.R. (1980). New map of bacteriophage lambda DNA. *Journal of Virology* 33(1) : 390-400.

DRAPER, J.; SCOTT, R. (1988). The isolation of plant nucleic acids. In: *Plant genetic transformation and gene expression: a laboratory manual* Edited by J. Draper, R. Scott, P. Armitage, and R. Walden. Blackwell Scientific Publications, Oxford.

DRETZEN, G.; BELLARD, M.; SASSONE-CORSI, P.; CHAMBON, P. (1981). A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. *Analytical Biochemistry* 112 : 295-314.

DUGAICZYK, A.; BOYER, H.W.; GOODMAN, H.M. (1975). Ligation of a Eco RI endonuclease-generated DNA fragments into linear and circular structures. *Journal of Molecular Biology* 96 : 171-184.

DYNAN, W.S. (1986). Promoters for housekeeping genes. *Trends in Genetics* 2 : 196-197.

DYNAN, W.S. (1989). Modularity in promoters and enhancers. *Cell* 58 : 1-4.

DYNAN, W.S.; TJIAN, R. (1985). Control of eukaryotic messenger rna synthesis by sequence-specific dna binding proteins. *Nature* 316 : 774.

FERNANDEZ-RENAU, D.; LOMBARDERO, M.; NIETO, A.(1984). Glucocorticoid-dependent uteroglobin synthesis and uteroglobin mRNA levels in rabbit lung explants cultured in vitro. *European Journal of Biochemistry* 144 : 523-540.

FLACH, G.; JOHNSON, M.H.; BRAUDE, P.R.; TAYLOR, R.A.S.; BOLTON, V.N. (1982). The transition from maternal to embryonic control in the 2-cell mouse embryo. *The EMBO Journal* 1(6) : 681-686.

FLAMING, D.G.; BROWN, K.G. (1982). Micrpipette puller design: form of the heating filament and effects of filament width on tip length and diameter. *Journal of Neuroscience Methods* 6 : 91-102.

FROMM, M.; BERG, P. (1983). Simian virus 40 early- and late- region promoter functions are enhanced by the 72-bp repeat inserted at distant locations and inverted orientations. *Molecular & Cellular Biology* 3 : 991-999.

GALLIE, D.R. (1991). The CAP and Poly(A) Tail function synergistically to regulate mrna translational efficiency. *Genes & Development* 5 : 2108-2116.

GASSER, S.M.; LAEMMLI, U.K. (1987). A Glimpse at Chromosomal Order. *Trends in Genetics* 3 : 16-22.

GETZENBERG, R.H.; PIENTA, K.J.; WARD, W.S.; COFFEY, D.S. (1991). Nuclear structure and the three-dimensional organization of DNA. *Journal fo Cellular Biochemistry* 47 : 289-299.

GIRVITZ, S.C.; BLACCHETTI, S.; RAINBOW, A.J.; GRAHAM, F.L. (1980). A rapid and efficient procedure for the purification of DNA from agarose gels. *Analytical Biochemistry* 106 : 492.

GOSWAMI, A.; FEIGELSON, M. (1974). Differential regulation of a low molecular weight protein in oviductal and uterine fluid by ovarian hormones. *Endocrinology* 95 : 669-680.

GROSS, D.S.; GARRARD, W.T. (1987). Poising chromatin for transcription. *Trends in Biochemical Sciences* 12 : 293-297.

GROSVELD, G.C.; DE BOER, E.; SHEWMAKER, C.K.; FLAVELL, R.A. (1982). DNA sequences necessary for transcription of the rabbit b-globin gene *in vivo*. *Nature* 295 : 120-126.

HAGEN, G.; WOLF, M.; KATYAL,S.L.; SINGH,G.; BEATO, M.; SUSKE, G. (1990). Tissue specific expression and 5'-flanking gene region of the rat Clara cell 10 kDa protein; comparison to rabbit uteroglobin. *Nucleic Acids Research* 18 : 2939-2945.

HAMM, J.; MATTAJ, I.W. (1990). Monomethylated cap structures facilitate RNA export form the nucleus. *Cell* 63 : 109-118.

HANAHAN, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* 166 : 557-580.

HANAHAN, D. (1988). Dissecting multistep tumorigenesis in transgenic mice. *Annual Review Genetics* 22 : 479-519.

HANAHAN, D.; JESSEE, J.; BLOOM, F.R. (1991). Plasmid transformtion of *Escherichia coli* and other bacteria. *Methods in Enzymology* 204 : 63-113.

HAYES, J.J.; WOLFFE, A.P. (1992). The interaction of transcription factors with nucleosomal DNA. *BioEssays* 14(9) : 597-603.

HERSHEY, J. W. B. (1991). Translation control in mammalian cells. *Annual Review of Biochemistry* 60 : 717-755.

HICKFORD, J. G. H. (1991). Personal communication.

HOGAN, B.; COSTANTINI, F.; LACY, E. (1986). Manipulating the mouse embryo: A Laboratory Manual Cold Spring Harbor Laboratory, USA.

INGRAHAM, H.A.; CHEN, R.; MANGALAM, H.J.; ELSHOLTZ, H.P.; FLYNN, S.E.; LIN, C.R.; Simmons, D.M.; Swanson, L.; Rosenfield, M.G. (1988). A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* 55 : 519-529.

ISH-HOROWICZ, D.; BURKE, J.F. (1981). Rapid and efficient cosmid cloning nucleic acids. *Research* 9 : 2989-2998.

JANTZEN, K.; FRITTON, H.P.; IGO-KEMANES, T.; ESPEL, E.; JANICH, S.; CATO, A.C.B.; MUGELE, K.; BEATO, M. (1987). Partial overlapping of binding sequences for steroid hormone receptors and DNase I hypersensitive sites in the rabbit uteroglobin gene. *Nucleic Acids Research* 15 : 4535-4552.

JOHNSON, P.F.; MCKNIGHT, S.L. (1989). Eukaryotic transcriptional regulatory proteins. *Annual Review of Biochemistry* 58 : 799-839.

JONES, N. (1990). Transcriptional regulation by dimerization: Two sides to an incestuous relationship. *Cell* 61 : 9-11.

KAY, E.; FEIGELSON, M. (1974). An estrogen modulated protein in rabbit oviductal fluid. *Biochemica Biophysica Acta*. 271 : 436-444.

KENNEDY, M. (1989). Personal communication.

KOZAK, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs nucleic acids. *Research* 12(2) : 857-872.

KOZAK, M. (1987). At least six nucleotides preceding the AUG Initiator codon enhance translation in mammalian cells. *Journal of Molecular Biology* 196 : 947-950.

KRISHNAN, R.S.; DANIEL, J.C.(1967). Blastokinin: inducer and regulation of blastocyst development in rabbit uterus. *Science* 158 : 490-492.

LATCHMAN, D.S. (1990). Review article: Eukaryotic transcription factors. *Biochemical Journal* 270 : 281-289.

LIEW, O. (1992). Personal communication.

LIN-CHAO, S.; CHEN, W.-T.; WONG, T.-T. (1992). High copy number of the pUC plasmid results from a rom/rop-suppressible point mutation in RNA II. *Molecular Microbiology* 6(22) : 3385-3393.

LIU, Z.-G.; SCHWARTZ, L.M. (1992). An efficient method for blunt-end ligation of PCR products. *BioTechniques* 12(1) : 28-30.

MCKNIGHT, S.; TJIAN, R. (1986). Transcriptional selectivity of viral genes in mammalian cells. *Cell* 46 : 795-805.

MANYAK, M.J.; KIKIKAWA, T.; MUKERJEE, A.B. (1988). Expression of a uteroglobin-like protein in human prostrate. *Journal of Urology* 140 : 176-182.

MITCHELL, P.J.; TJIAN, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 245 : 371-378.

MODRICH, P.; LEHMAN, I.R. (1970). Enzymatic joining of polynucleotides IX. A simple and rapid assay of polynucleotide joining (liages) activity by measurement of circle formation from linear deoxyadenylate-deoxythymidylate co-polymer. *The Journal of Biological Chemistry* 245(14) : 3626-3631.

MOOR, R.M.; POLGE, C.; WILLADSEN, S.M. (1980). Effect of follicular steroids on the maturation and fertilization of mammalian oocytes. *Journal of Embryological Experimental Morphology* 56 : 319-335.

MOORE, P.B. (1989). Elongation remodelled. *Nature* 342 : 127-128.

MULLIGAN, R.C.; BERG, P. (1981). Factors governing the expression of a bacterial gene in mammalian cells. *Molecular & Cellular Biology* 1(5) : 449-459.

NARAYAN, P.; ROTTMAN, F.M. (1992). Methylation of mRNA. *Advances in Enzymology* 65 : 255-285.

NELSON, C.; ALBERT, V.R.; ELSHOLTZ, H.P.; LU, L.I.-W.; ROSENFELD, M.G. (1988). Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. *Science* 239 : 1400-1405.

NEVINS, J.R. (1983). The pathway of eukaryotic mRNA formation. *Annual Review of Biochemistry* 52 : 441-466.

PABO, C.O.; SAUER, R.T. (1992). Transcription factors: Structural families and principals of DNA recognition. *Annual Review of Biochemistry* 61 : 1053-1095.

PAYNTON, B.V.; REMPEL, R.; BACHVAROVA, R. (1988). Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. *Developmental Biology* 129 : 304-314.

PIKO, L.; CLEGG, K.B. (1982). Quantitative changes in total RNA, total Poly(A), and ribosomes in early mouse embryos. *Developmental Biology* 89 : 362-378.

PROUDFOOT, N.J. (1989). How RNA polymerase 11 terminates transcription in higer eukaryotes trends in biochemical. *Sciences* 14 : 105-109.

QUINN, P.; BARROS, C.; WHITTINGHAM, D.G. (1982). Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *Journal of Reproduction & Fertility* 66 : 161-168.

RACOWSKY, C.; BALDWIN, K.V. (1989). *In vitro* and *in vivo* studies reveal that hamster oocyte meiotic arrest is maintained only transiently by follicular fluid, but persistently by membrana/cumulus granulosa cell contact. *Developmental Biology* 134 : 297-306.

RICHMOND, T.J.; FINCH, J.T.; RUSHTON, B.; RHODES, D.; KLUG, A. (1984). Structure of the nucleosome core particle at 7 angstroms resolution. *Nature* 311 : 532-537.

ROBERGE, M.; GASSER, S.M. (1992). DNA loops: structural and functional properties of scaffold-attached regions. *Molecular Microbiology* 6(4) : 419-423.

ROMIG, H.; FACKELMAYER, F.O.; RENZ, A.; RAMSPERGER, U.; RICHTER, A. (1992). Characterization of SAF-A, A novel nuclear binding protein from hela cells with high affinity for nuclear matrix/scaffold attachment DNA elements. *The EMBO Journal* 11(9) : 3431-3440.

SAMBROOK, J.; FRITSCH, E.F.; MANIATIS, T. (1989). Molecular cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, USA 2nd Edition.

SASSONE-CORSI, P.; DUBOULE, D.; CHAMBON, P. (1986). Transcriptional regulation by trans-acting factors. *Trends in Genetics* 2 : 215-219.

SAZER, S.; SCHIMKE, R.T. (1986). A Re-examination of the 5'-termini of mouse dihydrofolate reductase mRNA. *Journal of Biological Chemistry* 261(10) : 4685-4690.

SLATER, E.P.; REDEUIHL, G.; THEIS, K.; SUSKE, G.; BEATO, M. (1990). The uteroglobin promoter contains a noncanoical estrogen responsive elements. *Journal of Molecular Endocrinology* 4 : 604-610.

SNEDECOR, G.W.; COCHRAN, W.G. (eds) 1982. *Statistical Methods* 7th Edition. The Iowa State University Press, USA.

STAUDT, L.M.; CLERC, R.G.; SINGH, H.; LE BOWITZ, J.H.; SHARP, P.A.; BALTIMORE, D. (1988). Cloning of a lymphoid-specific cDNA Encoding a protein binding the regulatory octamer DNA motif. *Science* 241 : 577-580.

STEIN, G.S.; LIAN, J.B.; DWORETZKY, S.I.; OWEN, T.A.; BORTELL, R.; BIDWELL, J.P.; VAN WIJNEN, A.J. (1991). Regulation of transcription-factor activity during growth and differentiation: Involvement of the nuclear matrix in concentrationand localization of promoter binding proteins. *Journal of Cellular Biochemistry* 47 : 300-305.

TORKELLI, T.; KRUSIUS, T.; JANNE, O. (1978). Uterine and lung uteroglobins in the rabbit. *Biochemica Biophysica Acta* 544 : 578-592.

VAN BLERKOM, J. (1981). Structural relationship and posttranslational modificaiton of stage-specific proteins synthesized during early preimplantation development in the mouse. *Proceedings of the National Academy of Sciences, USA* 78(12) : 7629-7633.

WASSARMAN, P.M. (1983). Oogenesis: Synthetic events in the developing mammalian egg. In. *Mechanisms and Control of Animal Fertilization.* Edited by J.F. Hartman. Academic Press, Inc. London.

WASYLYK, B.; DERBYSHIRE, R.; GUY, A.; MOLKO, D.; ROGET, A.; TEOULE, R.; CHAMBON, P. (1980). Specific *in vitro* transcription of conalbumin gene is drastically decreased by a single-point mutation in T-A-T-A box homology sequences. *Proceedings of the National Academy of Sciences, USA* 77(12) : 7024-7028.

WEISS, B.; JACQUEMIN-SABLON, A.; LIVE, T.R.; FAREED, G.C.; RICHARDSON, C.C. (1968). Enzymatic breakage and joining of deoxyribonucleic acid. VI Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. *The Journal of Biological Chemistry* 243 : 4543-4555.

XANTHOPOULOS, K.G.; MIRKOVITCH, J.; DECKER, T.; KUO, C.F.; DARNELL, JR, J.E. (1989). Cell-specific transcriptional control of the mouse DNA-binding protein mC/EBP. ***Proceedings of the National Academy of Sciences, USA*** 86 : 4117-4121.

XANTHOPOULOS, K.G.; PREZIOSO, V.R.; CHEN, W.S.; SLADEK, F.M.; CORTESE, R.; DARNELL, JR, J.E. (1991). The different tissue transcription patterns of genes for HNF-1, C/EBP, HNF-3 and HNF-4, protein factors that govern liver-specific transcription. *Proceedings of the National Academy of Sciences, USA* 88 : 3807-3811.

XU, L.; THALI, M.; SCHAFFNER, W. (1991). Upstream Box/TATA box order is the major determinant of the direction of transcription. *Nucleic Acids Research*. 19(24) : 6699-6704.

YAMAMOTO, K.R. (1986). Hormone-dependent transcriptional enhancement and its implications for mechanisms of multifactor gene regulation. Edited by L. Bogorad. In 43*rd Symposium of the Society for Developmental Biology*. Molecular Developmental Biology Alan R. liss, Inc.

APPENDIX I
Cloning Calculations

Calculations of vector to insert ratios for ligations (Tables 2.1-2.3)
Vector to insert ratios were calculated for ligations according to Dugaiczyk *et al.* (1975) and Sambrook *et al.* (1989).

Calculations for Table 2.1

Fragment	Size (kbp)	Molecular Weight	J (ends/ml)
pSP65 Vector	3.03 kp	2.0×10^6	2.19×10^{13}
LacZ Insert	3.6 kp	2.43×10^6	1.64×10^{13}

[DNA] to obtain $j:i$ =2 for insert = 1/2 X 51.1/ $(2.43 \times 10^6)^{3/2}$ M

$$= 6.7 \times 10^{-9}$$

A 10µl ligation reaction will contain:

insert: 6.7×10^{-9} M x 2.43×10^6 x 10µl
= 163 ng LacZ

vector: 6.7×10^{-9} M x 2.0×10^6 x 10µl
=134 ng pSP65

Calculations for Table 2.2

	Size (kbp)	Molecular Weight	J (ends/ml)
UG400 (Insert)	400 bp	5.55×10^5	1.5×10^{14}
pSPLacZ Insert	6600 bp	4.5×10^6	6.5×10^{12}

[DNA] to obtain a j/i of 2 = 1/2 x 51.1/ $(5.55 \times 10^5)^{3/2}$
$=6.18 \times 10^{-8}$

A 10µl ligation reaction will contain:

Insert: 10ul x 5.55 x 10^5 x 6.18 x 10^{-8}
= 343 ng UG400

Vector: 10µl x 4.5 x 10^6 x 6.18 x 10^{-8}
= 2.8 ug pSPLacZ

Calculations for Table 2.3

	Size (kbp)	Molecular Weight	J (ends/ml)
UG3.3 (Insert)	3.3 kbp	2.2 x10^6	1.9 x10^{13}
pSPLacZ Insert	6.6 kbp	4.5 x10^6	6.5 x10^{12}

[DNA] to obtain a j/i ratio of 2 = 1/2 x 51.1 / (2.2 x 10^6)$^{3/2}$
= 7.8 x 10^{-9}

For a 10µl ligation reaction:

Insert: 7.8 x 10^{-9} x 2.2 x 10^6 x 10µl
= 172 ng UG3.3

Vector: 7.8 x 10^{-9} x 4.5 x 10^6 x 10µl
= 351 ng pSPLacZ

Appendix II
Microinjection Data

Date	Microinjection Data						Culture Data		Control Data	
	No. Don.	Pl. Don.	# Ova Coll.	No. Fert.	No. Inj.	No. Surv.	No. 2-Cell	No. 1-Cell	No. Cult	No. 2-Cell
Preliminary Trials (P610ZA)										
5.06.92	4	4	57	54	26	11	6	4	15	15
9.06.92	3	3	38	31	23	21	11	10	8	6
11.06.92	4	0								
17.06.92	3	3	48	26	20	16	12	3	5	5
19.06.92	3	1	37	20	15	11	8	2	5	5
pUG3.3LACZ										
16.07.92	5	2	46	25	21	12	9	3	4	4
15.08.92	4	4	76	45	39	33	24	8	4	3
16.08.92	3	3	77	70	54	42	41	0	6	6
17.08.92	3	3	11	10	5	5	3	2	2	2
p610ZA										
19.07.92	5	4	86	71	54	36	33	3	6	4
4.08.92	3	3	52	38	31	23	16	7	7	7
8.08.92	3	2	47	38	32	29	23	6	6	5
11.08.92	3	0								
12.08.92	4	2	37	33	29	24	10	12	4	4
pSPLACZ										
25.07.92	4	4	112	48	43	26	16	10	5	5
1.08.92	3	2	56	49	42	37	31	6	7	6
5.08.92	3	3	57	47	41	36	25	9	6	6
19.08.92	3	1	21	18	18	15	11	4	0	0
pUG400LACZ										
26.07.92	4	4	96	55	49	47	41	5	6	6
29.07.92	3	2	47	33	27	23	19	3	6	6
18.08.92	3	2	62	60	48	43	40	3	4	4
20.08.92	3	1	31	30	24	22	19	3	4	4

DATE	Stained				Unstained		
	2-Cell		1-Cell	Dead	2-Cell	1-Cell	Dead
	1/2	2/2					
Preliminary Trials (p610ZA)							
5.06.92	0	6	4	0	0	0	14
9.06.92	1	7	10	0	0	0	0
11.06.92							
17.06.92	0	3	0	0	0	1	1
19.06.92	0	7	1	0	1	1	2
pUG3.3LacZ							
16.07.92	0	0	0	0	9	3	9
15.08.92	0	0	0	0	24	8	3
16.08.92	0	0	0	0	41	0	4
17.08.92	0	0	0	0	3	2	0
p610ZA							
19.07.92	1	28	3	0	1	0	17
4.08.92	0	10	6	0	6	0	3
8.08.92	2	18	5	0	3	1	0
11.08.92							
12.08.92	0	9	8	0	1	4	4
pSPLacZ							
25.07.92	0	0	0	0	14	10	17
1.08.92	0	0	0	0	31	6	5
5.08.92	0	0	1	0	25	8	2
19.08.92	0	0	0	0	11	4	0
pUG400LacZ							
26.07.92	0	0	0	0	41	5	2
29.07.92	0	0	0	0	19	3	5
18.08.92	0	0	0	0	40	3	2
20.08.92	0	0	0	0	19	3	2